University of Alberta

Interactions Between Tumor Necrosis Factor Alpha and Vasoactive Pathways During Estrogen Deficiency

by

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ABSTRACT .....

Aging is associated with alterations in the mechanisms of vascular homeostasis. In women, the decline of the ovarian function with menopause has been epidemiologically associated with higher risk of developing cardiovascular disease. Moreover, these observations are supported by experimental studies that indicate that menopause is associated with alterations in vascular function, and suggests a role of a lack of ovarian estrogens on these cardiovascular alterations. It has been hypothesized that nflammatory factors play a role in the pathogenesis of cardiovascular disorders. Estrogen has modulator effects on immune function and its deficiency is associated with alterations in inflammatory pathways. Thus, interaction between estrogen and inflammatory factors may play a role in the pathogenesis of vascular dysfunction after menopause. The primary hypothesis of this thesis is that Tumor Necrosis Factor Alpha (TNFα), an inflammatory cytokine, is a mediator of vascular dysfunction in the state of estrogen deficiency. Our results show that in a model of female aging, estrogen deficiency is associated with an increase in circulating levels of bioactive TNFα, which results in a decrease in vasodilation and enhances vasoconstriction by reducing endothelial nitric oxide availability and increasing Angiotensin II modulation of vasoconstriction. Moreover, TNFα-Angiotensin II interaction was further explored in experiments conducted in isolated endothelial cells in culture. Our experiments indicate that Angiotensin II increases the formation of TNFα by endothelial cells, which in turn mediates the release of matrix metalloproteinase–2 induced by Angiotensin II. The results of the studies presented in this thesis suggest that interactions between TNFα altering NO

and ANG II pathways are involved in the development of vascular dysfunction in estrogen deficiency.

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LIST OF ABBREVIATIONS AND UNITS

The following abbreviations, definitions and units have been used throughout this thesis.

°C .....degrees Celsius

-/-.....homozygous knockout mice

-/+.....heterozygous knockout mice

+/+.....wild type (control) mice

ACE.....angiotensin converting enzyme

ANG II .....angiotensin II

ANOVA .....analysis of variance

AT_1R .....angiotensin 1 receptor

AT_2R .....angiotensin 2 receptor

ATP.....adenosine triphosphate

BH_4.....tetrahydrobiopterin

BK.....bradykinie

BP.....blood pressure

BSA.....bovine serum albumin

Ca^{2+} .....calcium ion

$CaCl_2$.....calcium chloride

CRP..... .....c-reactive protein

cAMP .....cyclic adenosine monophosphate

cGMP .....cyclic guanidine monophosphate

cDNA .....complementary deoxyribonucleic acid

C_T .....threshold cycle

CVD..... .....cardiovascular disease

DAG.....diacylglycerol

DMEM .....Delbecco's modified eagles medium

DMSO.....dimethyl sulphoxide

DNA.....deoxyribonucleic acid

dNTP.....deoxy nucleic acid triphosphate

EC.....endothelial cells

EC_{50}.....effective dose eliciting 50% response

EC_{80}.....effective dose eliciting 80% response

ECL.....enhanced chemiluminescence

EDHF .....endothelial-derived hyperpolarizing factor

EDTA.....ethylenediaminetetraacetic acid di-sodium salt

ERK..... .....extracellular signal-regulated kinase

eNOS.....endothelial nitric oxide synthase

et al.....*et alii* (Latin, 'and others')

g.....gram(s)

H_2O .....water

H_2O_2 .....hydrogen peroxide

HBSS.....Hank's Balanced Salt Solution

HEPES .....hydroxyethyl-1-piperazineethanesulfonic acid

HUVEC.....human umbilical vein endothelial cells

i.e......*id est* (Latin, 'that is')

IgG .....Non-specific mouse immunoglobulin G

IHC.....immunohistochemistry

IP_3.....inositol triphosphate

IL-6 .....interleukin-6

KCl.....potassium chloride

kD.....kilodalton

l .....litre(s)

LDH .....lactose dehydrogenase

L-NAME.....N^G-nitro-L-arginine methyl ester

m .....meter(s)

M.....moles·l^{-1}

MCP-1.....Monocyte chemoattractant protein-1

$MgCl_2$.....magnesium chloride

min .....minute(s)

MMPs.....matrix metalloproteinases

MMP-2.....matrix metalloproteinase-2

MnTBAP.....Mn(III)tetrakis(4-benzoic acid) porphyrin chloride

mRNA.....messenger ribonucleic acid

MS-PPOH .....N-methylsulphonyl-6-(2-proparglyloxyphenyl) hexanamide

n.....number of animals or experiments

N/A.....not applicable

NaAc .....sodium acetate

NaCl.....sodium chloride

NAD(P)H .....coenzyme nicotinamide adenine dinucleotide (phosphate)

NaN_3.....sodium azide

NCH .....Nottingham city hospital

NFκB.....nuclear factor kappa B

N-terminal .....amino-terminal

NO.....nitric oxide

NOS.....nitric oxide synthase

O_2 .....molecular oxygen

O_2^- .....superoxide anion

OD.....optical density

OH^- .....hydroxyl radical

p value.....probability (of incorrectly rejecting the null hypothesis)

PBS .....phosphate buffered saline

PCR.....polymerase chain reaction

PDTC .....pyrroline dithiocarbamate

PE.....phenylephrine

PEG-SOD.....polyethylene glycolated superoxide dismutase

pH.....logarithmic unit measuring acidity

PGI_2.....prostacyclin

PKA.....protein kinase A

PKC.....protein kinase C

PKG.....protein kinase G

PLA_2.....cytosolic phospholipase A_2

RAS.....renin angiotensin system

RIP .....Receptor Interacting Protein

RNA .....ribonucleic acid

ROS.....reactive oxygen species

RT .....reverse transcription

s.....second(s)

SD .....standard deviation of the mean

SDS .....sodium dodecylsulphate

SEM .....standard error of the mean

SNP .....sodium nitroprusside

SOD.....superoxide dismutase

TBS .....tris-buffered saline

TIMP-2.....tissue inhibitor matrix metalloproteinase-2

TNFα .....tumor necrosis factor-alpha

TRADD.....tumor necrosis factor receptor associated Death Domain

TRAF .....tumor necrosis factor receptor associated factor

Tris-Cl.....tris(1)-amino methane hydrochloride

tRNA .....transfer ribonucleic acid

V.....volts

VCAM.....vascular cellular adhesion molecule

VSMC..... .....vascular smooth muscle cells

Mathematical prefixes

k.....kilo (10^3)

c.....centi (10^{-2})

m .....milli (10^{-3})

μ .....micro (10^{-6})

n.....nano (10^{-9})

p.....pico (10^{-12})

CHAPTER 1. BACKGROUND TO THE DISSERTATION

1.1 INTRODUCTION

Despite advances in treatment during the last few decades, cardiovascular disease (CVD) remains as one of the main causes of death and morbidity globally (1). In postmenopausal women, CVD is the primary cause of death in developed countries (2). Moreover, the absolute number of women dying each year will continue to rise due to the aging of the population. Indeed, since the population over age 60 is expected to double during the next two decades the number of women affected by CVD will also increase. For instance, in the United States 60% of women over age 50 are hypertensive (2). Furthermore, in a recent analysis it was predicted that by the year 2020, at least 80% of women over the age of 50 across different populations, will develop hypertension (3). Therefore, the detection and understanding of the early vascular alterations that result in vascular disease in women during aging are essential to design strategies of prevention, and are the main subject of this thesis.

1.2 AGING AND CHANGES IN VASCULAR FUNCTION IN WOMEN.

One of the key physiological events in a women's life that marks an epidemiological transition from a low to a high-risk profile for CVD is the decline of the ovarian function with menopause. Several observational studies have shown that post-menopausal women are at higher risk for developing vascular disease compared with pre-menopausal women (4-7). Moreover, when compared with men of a similar age, premenopausal women have lower incidence of adverse cardiovascular events including coronary artery disease (8),

hypertension (2) and stroke (9). However, this protective trend is lost after menopause. In addition, women who undergo surgical menopause without estrogen replacement have two times the risk of coronary artery disease compared with cycling premenopausal women (10). Results from the third National Health and Nutrition Examination Study (NHANES III) found that the rise in blood pressure with age tends to be steeper in postmenopausal women compared with premenopausal women (Figure 1.1)(11). Moreover, compared with men, premenopausal women have a lower prevalence of hypertension, however the prevalence of hypertension is higher in postmenopausal women compared with men (Figure 1.1)(11). Furthermore, longitudinal studies have also shown that in peri and postmenopausal women the increase in blood pressure with age is greater compared with premenopausal women (2).

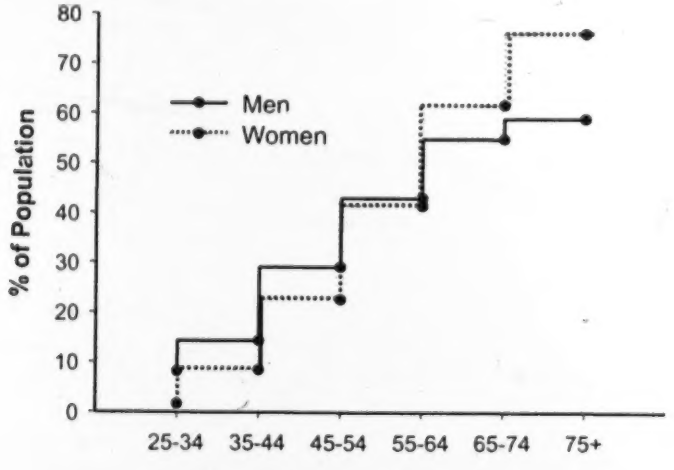

Figure 1.1 **Prevalence of Hypertension in USA by aged and gender. 1988-1994** Results from the National High Blood Pressure Education Program. Modified from *Wolz et al* (11).

Almost a decade ago, Celermajer et al reported that the percentage of vasodilation of the brachial artery to reactive hyperemia (flow-mediated dilation, which is an endothelium-dependent response) was progressively impaired with aging in both men and women (12), whereas the responses to glycery trinitrate (endothelium-independent) were not altered by age (Figure 1.2). The authors found that in men flow-mediated dilation was preserved in subjects aged < or = 40 years but declined progressively thereafter. However, in women, flow-mediated dilation was stable until the early 50s, after which it begins to decrease, suggesting that the decline in flow-mediated vasodilation in women was related to the onset of menopause (12).

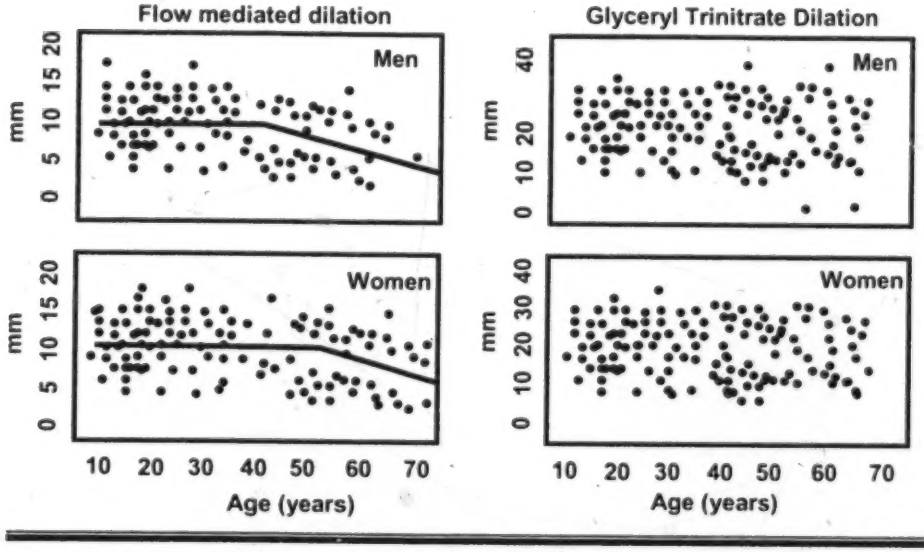

Figure 1.2 **Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women.** Modified from *Celermajer et al* **(12)**.

Furthermore, almost simultaneously with the work of Celermajer et al, it was also reported that endothelium-dependent vasodilation of coronary arteries (agonist-induced) in postmenopausal women was enhanced after estrogen replacement (13, 14). Further studies also reported that long-term hormone replacement was associated with an increase in circulating levels of nitrites and nitrates (an indirect measure of nitric oxide (NO) production), suggesting that the improvement in vasodilation after hormone replacement was related to an increase in NO production (15, 16).

These landmark studies along with the epidemiological observations suggest that the lack of the ovarian hormones after menopause, in particular estrogen, is associated with alterations in vascular function. In premenopausal women, 17β estradiol is the main bioactive circulating estrogen. Serum levels of estradiol range from about 100 pg/mL in the follicular phase to about 600 pg/mL at the time of ovulation. After menopause, estradiol levels dramatically fall and are similar to or lower than those in men of similar age (5 to 20 pg/mL).

Estrogen has been found to have multiple effects on the cardiovascular system (reviewed in (17)), and several studies have shown that it is protective against vascular injury (18-20) and atherosclerosis (21, 22). Nevertheless most of these studies have focused on the direct role of estrogen on vascular function and the contribution of other factors to vascular dysfunction during estrogen deficiency is poorly understood. For instance, estrogen has well-known actions on the plasma lipid profile that may account for part of the protective effects of endogenous estrogens on cardiovascular mortality (23). Therefore, it is likely that other factors influenced by the lack or estrogen and/or other ovarian hormones play a role in the cardiovascular alterations associated with

menopause. Indeed, recent clinical trials of hormone replacement have found that estrogen replacement was associated with higher risk of acute cardiovascular events (24-26), and underscored the need for further understanding of the mechanisms associated with vascular dysfunction after menopause.

Aging is associated with alterations in both the endocrine and the immune systems (27), and it is likely that interactions between both regulatory systems are involved in the physiological changes associated with senescence, including reproductive and cardiovascular senescence (27). In fact, estrogen has regulatory effects on immune function and its deficiency is associated with autoimmune disorders, and alterations in inflammatory pathways (section 1.4). During the last decade clinical and experimental observations have revealed the importance of inflammatory factors in the pathophysiology of cardiovascular disease (section 1.3). Thus, interplay between estrogen and inflammatory factors could play a role in the alterations of vascular function during menopause. However, whether inflammatory factors are involved in the vascular dysfunction associated with estrogen deficiency is largely unknown and it is the focus of this thesis. Specifically, I evaluated the role of Tumor Necrosis Factor alpha (TNFα), a pro-inflammatory cytokine in vascular function in a model of aging in female rats. The likely role of TNFα in aging female rats was studied using isolated resistance arteries focusing on the NO and Angiotensin (ANG) II pathways. Alterations in these vasoactive pathways have been strongly associated with the development of cardiovascular disease.

To provide background for the dissertation, I will first overview pathways of vascular function focusing on the role of resistance arteries, and their alterations during the state of estrogen deficiency. Next, the role of inflammation in vascular disease as well

as the effects of estrogen on immune function will be reviewed. This will be followed by a review of the physiology of TNFα, which will then lead to my working hypothesis that interactions between TNFα altering regulatory pathways of endothelial and vascular function leads to vascular dysfunction in the state of estrogen deficiency.

1.3 NITRIC OXIDE, VASCULAR FUNCTION, AND ESTROGEN

Peripheral resistance is an important determinant of blood pressure. Most of the resistance to blood flow occurs at the level of small arteries and arterioles (28). Based on Poiseuille's law (hydraulic resistance equation), it has been demonstrated that resistance to flow in small arteries increases with the fourth power of the artery radius (29). Therefore, small changes in the vascular lumen have a large impact on resistance to flow. Importantly, small arteries (so called resistance-size arteries), which are composed mostly of smooth muscle fibers in the walls, have a greater capacity to modify their vascular lumen (e.g. from complete dilation to complete vasoconstriction) compared with large elastic arteries. Hence, dysfunction of small arteries may result in exaggerated vascular resistance and hypertension. The degree of dilation of an artery is determined by its tone, which is generated by the active contraction of vascular smooth muscle cells. The stimulus that determines basal vascular tone is unclear, however, it is modulated by several vasoactive stimuli such as endothelial-derived or systemic circulating vasoactive factors, local metabolic factors and autonomic nervous stimulation. The net effect of these factors determines vascular tone and thus, the distribution of blood to tissues.

1.3.1 Endothelial modulation of vascular function

The endothelium that covers an area of more than 5000 m^2 in an adult person (30), was described in the 19th century by Von Recklinghausen as an inner cellular lining of blood vessels, which was thought to function only as a physical barrier between the blood and vascular tissue. As a passive layer, endothelial cells regulate the diffusion of several substances between tissues and blood. Moreover, central to the dissertation of this thesis, is the ability of endothelium-derived factors to modulate the contractility of underlying vascular smooth muscle cells, and thus, to control vascular tone. Thanks to a "Nobel mistake" of his technician (Nobel Lecture, 1998), who unintentionally removed the endothelial layer of aortic preparations, Robert Furchgott reported in 1980 that the endothelium was the source of a relaxing factor, termed endothelial derived relaxing factor (EDRF) (31). A few years later it was found that EDRF was NO (32).

NO is a lipophilic gas that is able to diffuse into the subendothelial space and enter the underlying vascular smooth muscle cells (VSMC) (33). NO production in endothelial cells is stimulated by several humoral factors such as growth factors, hormones (including estrogen) and vasoactive mediators such as bradykinin, Angiotensin II, etc. (32, 34). Moreover, NO release is also induced by mechanical forces such as shear stress caused by the friction of the blood with the vascular wall, which constitutes the primary physiological mechanism that maintains the basal release of NO. Importantly, basal production of NO is involved in the regulation of peripheral resistance and its inhibition is associated with an elevation of blood pressure (35, 36).

Physiological levels of shear stress may vary within the vascular system from 10 dynes/cm^2 in the aorta to ~ 70 dynes/cm^2 in the arterioles. In mesenteric arteries, wall

shear stress has been estimated to be ~ 60 dynes/cm^2 (37). Shear stress plays a role in the control of vascular structure and function, including regulation of vascular tone, remodeling, hemostasis and vascular inflammation (38), (39, 40). In fact, some observations suggest that areas of the vascular tree that are not exposed to laminar flow but to "disturbed flow" are more likely to develop atherosclerosis (41). Most of the protective effects of shear stress on vascular function seem to be mediated by the formation of NO (42, 43).

NO is formed by the enzyme NO synthase (NOS), of which there are 3 isoforms: eNOS (Isoform III) that is found mainly in endothelial cells; iNOS (Isoform II), which is an inducible isoform and nNOS (neuronal; NOS I). eNOS is constitutively active and it is considered the key isoform that mediates the vasculoprotective effects of NO (44, 45). Shear stress regulates the expression of eNOS and there are shear stress response elements in the gene promoter region of eNOS (46).

NO is synthesized from L-arginine through oxidation of molecular oxygen that requires NADPH as an electron donor and tetrahydrobiopterin (BH4) and flavin mono/dinucleotides as cofactors (47), as well as heme and calmodulin (CaM), which are involved in the transference of electrons from NADPH for NO generation (48). Interestingly, in the absence of the substrate arginine or the cofactor BH4, NOS also generates superoxide anion (NOS uncoupling) (49). NOS uncoupling has been described for all NOS isoforms (50, 51).

eNOS is localized in specialized invaginations of the plasma membrane named caveolae, which are highly expressed in endothelial cells (52). eNOS is inhibited by interactions with caveolin-1 that is a membrane protein located in caveolae (53).

Moreover, in caveolae eNOS also interacts with the cationic aminoacid transproter CAT-1, responsible for the transfer of arginine across the membrane (54). In the presence of agonists that increase cytosolic calcium (55), CaM associates with eNOS, and caveolin-1 is displaced, releasing eNOS from the plasma membrane to the cytosol (56) (Figure 1.3). Thus, factors that induce an increase in intracellular calcium such as bradykinin and estrogen increase eNOS activity. For instance, some G-protein linked receptors activate phospholipase C to increase cytoplasmic calcium and stimulate eNOS activity (56).

Shear stress also induces eNOS translocation to the cytosol (57). However, eNOS activation by shear stress seems to involve calcium dependent and independent mechanisms (57) (Figure 1.3). Shear stress Ca^{2+}/CaM-dependent eNOS activation lasts few seconds, and is mediated by GTP-binding proteins (58). However, Ca^{2+}-independent eNOS activation (long lasting) is independent of GTP-binding proteins (59). Evidence suggests that the phosphorylation of eNOS at S1179 by a sequential activation of phosphoinoside 3-kinase (PI3K) and Akt pathway is the underlying mechanism by which shear stress stimulates NO production in a Ca^{2+}/CaM-insensitive manner (60, 61). However, protein kinase A (PKA) activation has also been shown to mediate shear stress-induced eNOS activity by phosphorylation at the S1179 residue (62). Although the upstream event to shear stress-induced PI3/Akt activation is unclear, potassium channels, which would act as mechanotransducers within the plasma membrane have been postulated (63).

Interestingly, Hutchenson et al showed that disruption of the endothelial cytoskeleton decreases flow-induced eNOS activity but did not alter agonist-induced or calcium-dependent NO generation (64). The authors speculated that changes in tension of the

endothelial cytoskeleton caused by shear stress modulate eNOS activity (64). In this regard, it has been proposed that shear stress signalling is mediated by components of the cytoskeleton such as the integrins (65). In particular, β-1 integrins have been implicated in sensing shear stress in endothelial cells (63). Moreover, integrin-linked kinase, a known activator of Akt (66) that is associated with β-1 integrins, has been suggested as a mediator of shear stress induced eNOS activation (67). However, eNOS phosphorylation may have both stimulatory and inhibitory effects. For instance, protein kinase C (PKC) phosphorylation inhibits eNOS activity (68).

NO produced by eNOS acts on endothelial cells and also diffuses away in the luminal direction and within the vascular wall. In VSMC NO binds to the heme component of guanylate cyclase, leading to a consecutive increase in cyclic guanosine monophosphate (cGMP) from guanosine triphosphate, which results in activation of cGMP dependent protein kinases that reduce cytosolic calcium (Figure 1.3)(69, 70). Additional mechanisms have been proposed and their role may vary in arteries from different vascular beds (Figure 1.3): 1) Opening of Na^{2+}/K^{+} ATPase dependent K^{+} channels and thus accelerating K^{+} efflux leading to cell membrane hyperpolarization and extrusion of calcium ions; 2) Inhibition of calcium channels and reduction of calcium release from the sarcoplasmic reticulum, resulting in a decrease in intracellular calcium concentrations; 3) Inhibition of Rho-kinase that leads to reduced interaction of contractile proteins.

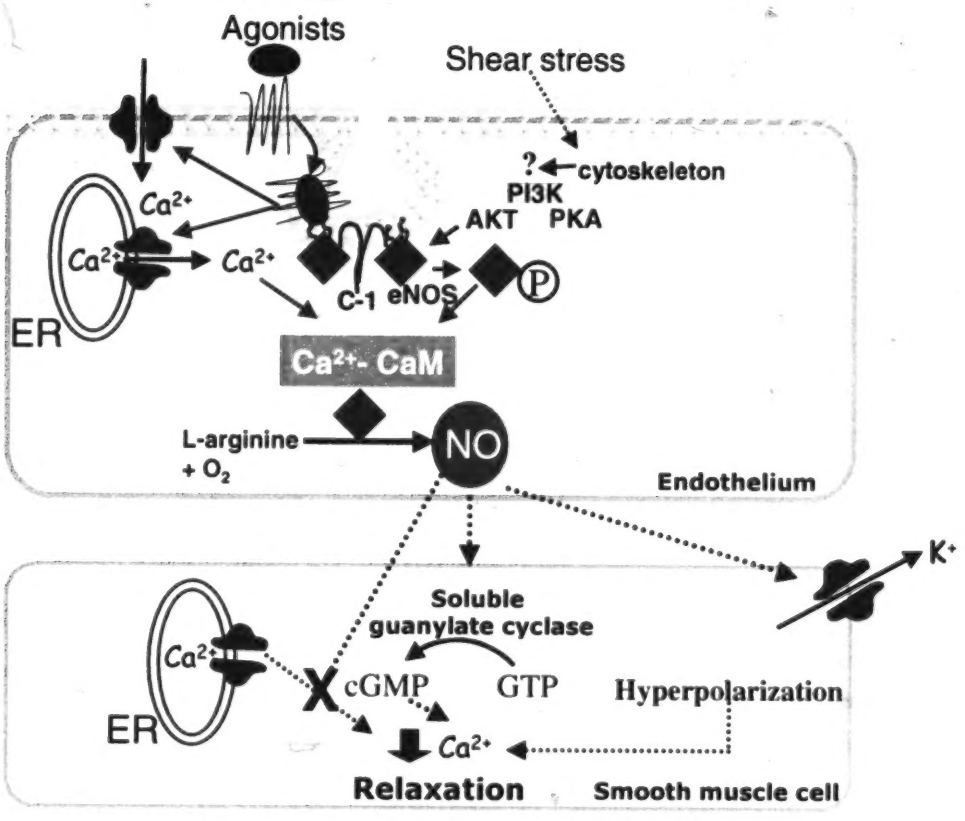

Figure 1.3 Mechanisms of eNOS activation and NO-induced vasodilation.

Agonist binding to a G-protein linked receptor (red-continuous arrows) results in activation of Ca^{2+} channels in the plasma membrane and endoplasmic reticulum (ER). Ca^{2+} induces separation of eNOS from caveolin-1 (C-1), binding of eNOS to calmodulin (CaM) and translocation to the cytosol where it produces NO from L-arginine. Shear stress (blue-dashed arrows) signals through the cytoskeleton and results in activation of PI3 kinase and then Akt and/or PKA.These kinases phosphorylate eNOS. NO produced in endothelial cells can induce relaxation of vascular smooth muscle cells by generation of cGMP, inhibiting the release of calcium from ER and by membrane repolarization with opening of potassium channels. Modified from *Govers et al*(56)

In addition, NO can also induce vasorelaxation indirectly by inhibition of the production of endogenous vasoconstrictors such as ANG II and endothelin-1 or scavenging of free radicals such as superoxide anion.

Besides vasorelaxation, NO modulates membrane permeability and fluidity, which facilitates the interaction between endothelial cells and the formed elements of the blood (71). Thus, a decrease in NO production by endothelial cells may result in increased membrane rigidity, leading to deceleration and trapping of formed elements in the capillary circulation (67), which may contribute to the increased peripheral resistance to flow. Moreover, NO inhibits platelet and monocyte adhesion to endothelial cells, which is triggered by several pro-atherogenic factors such as cytokines (72, 73). Moreover, NO is able to suppress apoptosis by inhibition of caspases (74, 75) as well as by upregulating the production of anti-apoptotic proteins (76, 77). In this regard, NO has been reported to inhibit TNFα-induced apoptosis by reducing the generation of ceramide (78). Reduction in NO availabiiity is associated with a decrease in dilation to flow or paradoxical constriction (79). Moreover, flow-mediated vasorelaxation is decreased in atherosclerotic vessels, and cardiovascular risk factors decrease the vasodilation to flow (80). Importantly, decreased flow-dependent relaxation is an independent predictor of future cardiovascular events (81). Thus, a decrease in endothelial-dependent vasodilation with estrogen deficiency is indicative of endothelial dysfunction, and understanding of the pathophysiological mechanisms is one of the aims of this thesis.

1.3.1.1 Estrogen, nitric oxide and endothelial function

Endothelial cells have functional estrogen receptors (ER) α and β (82), and accordingly, estrogen modulates many relevant endothelial cell functions, such as vascular tone, inflammation, and angiogenesis, among others. Estrogen is known to increase the production of nitric oxide (NO) through genomic and non-genomic mechanisms (17, 83).

Acute effects of estrogen (non-genomic) on NO release are mediated by calcium dependent mechanisms without altering eNOS expression (84). Although ERα has been primarily involved in this effect, acute eNOS activation can be also mediated by ERβ (85, 86). Acute eNOS stimulation by estrogen involves activation of MAPK kinases (87) and Akt (88). Interestingly, in studies conducted in plasma membranes isolated from ovine endothelials cells, estradiol was found to increase NOS activity 92% compared with basal levels in the absence of calcium and calmodulin. In the same preparations, a mixture of calcium, calmodulin and cofactors resulted in a 170% increase in NOS activity. However, estradiol did not further enhance NOS activity when administered with this mixture (89). Long-term exposure to estrogen up-regulates the expression of eNOS mRNA and protein in endothelial cells (90-93), it is primarily mediated by estrogen β receptors (94), and is associated with an increase in NO production.

In addition to the lack of estrogen-mediated NO production after menopause, additional mechanisms may also contribute to decrease NO availability during estrogen deficiency. In fact, estrogen can also increase NO availability by reducing the formation of free radicals in endothelial cells (95). Superoxide anion is involved in NO scavenging and in the subsequent formation of peroxynitrite, which in endothelial cells can induce the activation of proinflammatory transcription factors (96). Although estrogen does not

seem to scavenge superoxide anion itself, some studies have shown that it regulates the activity of NAD(P)H oxidase (92, 97, 98), which is one of the main sources of superoxide anion in the endothelium. In this regard, it has been reported that estrogen downregulates the expression of NAD(P)H oxidase induced by TNFα (99) or ANG II (92). Furthermore, estrogen prevents the destabilization of eNOS mRNA caused by TNFα (100). These observations indicate that estrogen deficiency is associated with a decreased in NO availability, which is mediated by multiple mechanisms. However, the contribution of other factors to the regulation of NO levels during estrogen deficiency is unclear, and it is one of the questions addressed in the second and third chapters of this thesis.

1.4 THE RENIN ANGIONTENSIN SYSTEM, VASCULAR FUNCTION AND ESTROGEN

In addition to the prominent changes in NO availability after menopause, there is evidence that other mechanisms, NO-independent, also contribute to cause vascular dysfunction after menopause. For instance, in resistance arteries of aged animals, estrogen deficiency is associated with an increase in the formation of endothelium-derived vasoconstrictors, which is reversed by estrogen replacement (101, 102). Moreover, Elhage et al. showed that inhibition of NO production did not influence the reduction of atherosclerosis achieved by estrogen replacement in *Apoe*$^{-/-}$ mice (103). Furthermore, three months of treatment with estrogen dramatically reduced atherosclerosis and significantly lowered blood pressure in eNOS and apoE deficient mice compared with animals treated with control pellets (104).

Angiotensin (ANG) II is a vasoconstrictor and pro-atherogenic factor that along with NO, plays an important role in the control of vascular homeostasis. Renin the first

component of the Renin-Angiotensin System (RAS), was discovered around 100 years ago by Tigerstedt and Bergman (105, 106). They found that extracts of kidney from rabbits caused elevation of blood pressure when injected into the circulation (105). Almost 40 years later Goldblatt et al, working on kidney ischemia and hypertension, turned the attention again to the pressor substances excreted by the kidney (106). By 1939 two independent research groups from Argentina and United States found that renin was a peptidase that produces the pressor peptide Angiotensin (a combination of the terms "hypertensin" and "angiotonin" used by each group respectively).

ANG II is a circulating vasoconstrictor with various other systemic actions such as stimulating the release of aldosterone from the adrenal gland. In the classical RAS, renin that is produced by the juxtaglomerular cells in the kidney cleaves circulating angiotensinogen (13 amino acids) in the plasma to form ANG I (a decapeptide), which is further converted into ANG II (an octapeptide) by the Angiotensin-Converting Enzyme (ACE), an enzyme with particularly high concentrations in the lung vasculature.

Effects of ANG II on the vascular wall are mediated by the ANG II type I (AT_1R) or type II (AT_2R) receptors located on vascular smooth muscle cells, fibroblasts and endothelial cells (reviewed in (107)). AT_1R and AT_2R are transmembrane glycoproteins linked to G-proteins with a 30% of sequence similarity. In rodents AT_1R exists as two distinct subtypes AT_{1A} and AT_{1B} that are 95% identical in their amino acid sequences. Both subtypes are also similar in terms of their ligand binding and signal transduction properties but differ in their tissue distribution. In rats, AT_{1A} is the isoform predominantly expressed in all tissues except in the adrenal and pituitary glands, where AT_{1B} is the most

abundant. AT_1R blockers such as Candesartan can pharmacologically block both receptors.

AT_1R is linked to $G_{q/11}$, and signaling through this receptor results in activation of several signaling pathways (reviewed in (108))(Figure 1.4). Rapid signaling events occur within seconds and are mediated by phospholipase C and c-Src tyrosine kinase activation. These events result on IP3 formation, calcium release and vascular contraction. Moreover, ANG II through activation of the ERK family of kinases can also induce phospholipase A activation (within minutes) and stimulate the production of arachidonic acid metabolites such as prostaglandins and lipoxygenase products that are involved in the control of vascular tone (108). Long-term vascular effects of ANG II such as cell growth, extracellular matrix deposition, and cell migration are mediated by activation of phospholipase D and tyrosine kinase cascades, which results in stimulation of redox sensitive pathways, protooncogen expression and in the production of growth factors and cytokines (108). Interestingly, ANG II is also able to induce signal transduction cascades typical of the pro-inflammatory cytokines such as TNFα. ANG II stimulates the production of monocyte chemotactic protein-1 (MCP-1) (109) and IL-6 in VSMC (110, 111). Moreover, in the endothelium, ANG II upregulates the expression of cellular adhesion molecules (112-114). Most of these effects seem to be mediated by AT_1R and involve free radical production and activation of NF-κB (109, 112)(Figure 1.4). Thus, NF-κB activation is an important mediator of ANG II-induced inflammation. Hence, in transgenic rats expressing human Angiotensinogen and human renin (AOGEN/renin), NF-κB is upregulated and its blockade substantially ameliorates the cardiac and vascular damage observed in this model (115, 116).

AT_2R is linked to Gi protein and activates p38 kinase pathways to oppose some of the effects of ANG II via AT_1R (117). AT_2R expression is high during fetal life and it seems to play a role in developmental processes and vascular remodeling (117). AT_2R is expressed in both VSMC and endothelial cells but its levels of expression vary depending on the vascular bed. In VSMC, AT_2R has pro-apoptotic effects (118), and in endothelial cells, AT_2R activation stimulates NO release from endothelial cells and results in vasodilation (119, 120). Indeed, AT_2R deficient mice have elevated blood pressure and increased vascular sensitivity to ANG II (121, 122).

The broad intracellular effects of ANG II and the evidence linking this hormone with cardiovascular disease led to the discovery of independent RAS in different tissues such as the brain, kidney, heart, adrenal gland and the vascular wall, where ANG II can be locally formed. Indeed, bilateral nephrectomy that eliminates renal renin from the circulation does not completely eliminate ANG II from the circulation (123). In the vascular wall, all components of RAS (renin, angiotensinogen, and ACE) can be found at both the level of mRNA and protein (124). In fact, ANG II formation has been demonstrated to occur in both isolated endothelial cells (125-128), and in VSMC (129, 130). Moreover, in VSMC up to 50% of ANG II generation may occur by renin-independent pathways (130).

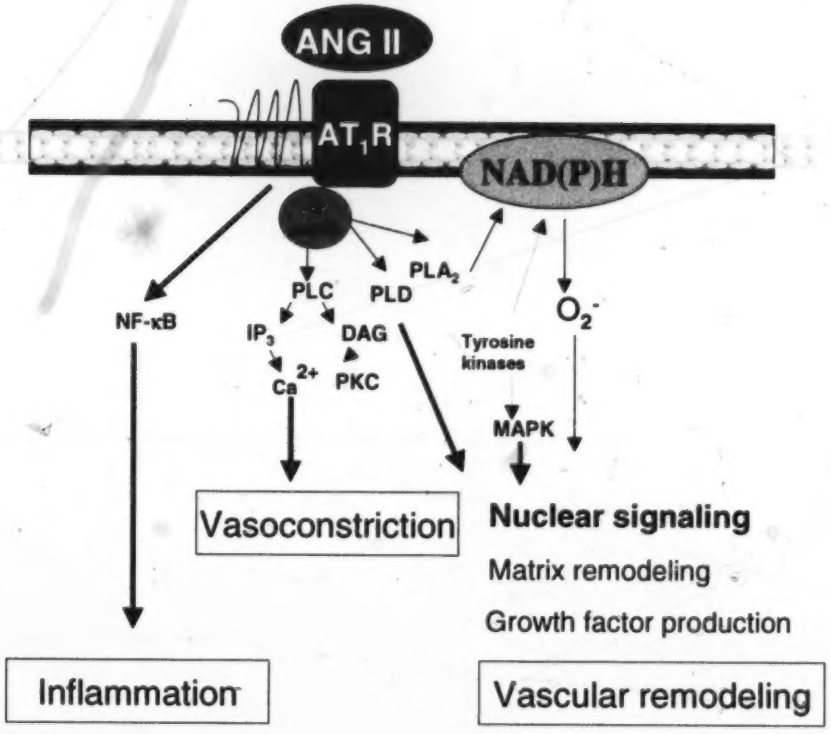

Figure 1.4 Ang II-mediated signaling events via AT_1R in vascular smooth muscle cells.

Binding of Ang II to the AT_1R stimulates activation of PLC and constitutes the immediate signaling events that result in contraction. Activation of PLA_2, PLD, tyrosine kinases, and MAP kinases with activation of NAD(P)H and generation of reactive oxygen species occurs within minutes and are the early signaling processes that lead to protein synthesis. *Modified from Touyz et al*(108).

The contribution of renin-independent systems to ANG II generation in endothelial cells is unclear. Experiments in isolated hindquarters of transgenic rats expressing human Angiotensinogen (that only can be cleaved by human renin), suggests that the cleavage of Angiotensinogen by infused renin occurs within the vascular wall, rather than in the lumen of blood vessels (131). Moreover, using similar experimental approaches, it has been shown that ANG II generation and its local pressor effects can also occur independently of circulating renin, by local renin-like activity in blood vessels (132). Furthermore, the level of ACE expression in vascular tissues correlates with the rate of local ANG II formation (131, 133) indicating that vascular ACE is a limiting step for ANG II formation, and that local generation of ANG II is important for the control of vascular tone.

Experiments on transgenic rats expressing both the human renin and human angiotensinogen, have shown that increased ANG II formation in vascular tissues is associated with hypertension, early vascular expression of adhesion molecules and leukocyte infiltration of the vascular wall along with increased oxidative stress and deposition of extracellular matrix proteins (134). These transgenic animals die at age 7 weeks from cardiac and renal failure and interestingly, blood pressure reduction does not protect from vascular alterations (116), which can be completely prevented by ANG II blockade (134). This model nicely summarizes most of the detrimental vascular effects attributed to ANG II such as vascular hyper-reactivity, hypertrophy, increased matrix remodeling and vascular inflammation. Hence, ANG II has been etiologically associated with major vascular diseases such as preeclampsia (135, 136), atherosclerosis (137) and hypertension (108, 138).

1.4.1 Estrogen deficiency and RAS activation

Several observations suggest that ANG II plays a role in the pathogenesis of vascular dysfunction during estrogen deficiency. Estrogen deficiency has been associated with increased levels of renin (139), ACE (140) and increased expression of AT_1R in vascular smooth muscle cells, which can be reverted by estrogen treatment (141, 142). Moreover, some studies have shown that infusion of 17β estradiol, but not of progesterone or 5-alpha-dihydroprogesterone suppresses the pressor responses to ANG II in nonpregnant animals (143, 144). Furthermore, *in vitro* experiments showed that 17-β estradiol decreases the contractility of human arteries to ANG II (145). These observations suggest that estrogen modulates the vascular effects of ANG II.

Accordingly, we recently demonstrated that in endothelial cells, estrogen decreases ANG II-mediated formation of peroxynitrite by reducing of AT_1R expression (92)(Appendix 3). In addition, in salt-sensitive rats, ovariectomy is associated with an increase in salt blood pressure sensitivity, which was linked to an increase in activation of ANG II (146). Altogether these results indicate that RAS activation along with an increase in the actions of ANG II, may be involved in the alterations of vascular function after menopause.

Estrogen deficiency is associated with increased vasoconstriction, which is in part mediated by an increase in the production of endogenous (produced by the vascular wall) vasoconstrictors (101, 102). Interestingly, endogenous ANG II modulates adrenergic constriction in different vascular beds including mesenteric arteries (147-149), aorta (120) and the rat caudal artery (150, 151). In those studies, vasoconstriction to adrenergic agonists such as phenylephrine (120, 147), norepinephrine (148, 152) or sympathetic

nerve stimulation (149) was decreased by ANG II receptor blockers (ARBs) or ACE inhibitors (149, 150, 153, 154). However, whether vascular ANG II may be involved on modulating vasoconstriction during the state of estrogen deficiency is unknown. This question is addressed in chapter four of this thesis.

Furthermore, the mechanisms involved in the alterations of RAS during estrogen deficiency are unclear. There is evidence that suggests that inflammation can activate RAS in the vascular wall (discussed in section 1.3.2). Thus inflammation leads to RAS activation, which in turn, switches on pro-inflammatory mechanisms in the vascular wall that could result in vascular damage when the defense mechanisms of the vascular wall are altered as it occurs during estrogen deficiency.

1.5 Inflammatory Factors, Vascular Function, And Estrogen

Blood vessels control the trafficking of immune cells to extravascular spaces, and therefore, interactions between immune cells and vascular cells play an important role in the control of the inflammatory response. Endothelial expression of adhesion such as Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Adhesion Molecule-1 (VCAM-1), and E-selectin facilitate the tethering of leukocytes to the endothelium and their migration to injured tissues. Moreover, endothelial cells and vascular smooth muscle cells are a source of chemoattractants such as MCP-1 and IL-8 that attracts and stimulates the migration of leukocytes to vascular and extravascular spaces. The expression of adhesion molecules, integrins and chemokines is stimulated by pro-inflammatory cytokines such as TNFα (Reviewed in (155)) On the other hand, there are anti-inflammatory mechanisms that modulate the responses to inflammatory factors in the

vasculature. Physiological shear stress is of particular importance in the protection of endothelial cells against inflammatory activation. For instance, flow induces NO production that inhibits NF-κB activation, MCP-1 expression, V-CAM-1, ICAM-1, and IL-6 production by endothelial cells (156-159). Moreover, anti-inflammatory cytokines such as IL-10, growth factors and peroxisome proliferator-activated receptors, may elicit also anti-inflammatory effects in the vascular wall (reviewed (160)). The imbalance between anti-inflammatory and pro-inflammatory stimuli may result in vascular inflammation and ultimately lead to vascular dysfunction.

1.5.1 Inflammation and cardiovascular disease

During the last two decades, mounting epidemiological and biological evidence indicate that inflammatory factors play an important role in the pathogenesis of vascular disease (161). Cytokines cause VSMC proliferation and migration to subintimal spaces (162), as well as alterations in extracellular matrix remodeling and collagen composition (163), which results in thickening and stiffness of arteries. Moreover, cytokine stimulated endothelial cells undergo functional alterations resulting in a pro-thrombotic and pro-inflammatory phenotype (activation) characterized by upregulation of adhesion molecules, production of chemotactic factors as well as the expression of procoagulant factors (164). Ultimately, these changes facilitate atherothrombosis and may result in cardiovascular disease.

In fact, subtle increases in serum levels of inflammatory factors are associated with higher risk of future cardiovascular events in apparently healthy individuals, including postmenopausal women (165-167). Accordingly, cardiovascular risk factors such as

oxidized low-density lipoprotein (oxLDL), smoking, and obesity are capable to stimulate the formation of "early inflammatory factors" such as IL-1 and TNFα from immune and non-immune cells (161). Thus, vascular cells are not only target for cytokines but also a source of inflammatory factors (164). These "primary cytokines" can alter vascular function directly or by stimulating the release of acute reactant proteins such as IL-6 and C-reactive protein (CRP) (161).

1.5.2 Tumor necrosis factor alpha (TNFα)

TNFα is a cytokine with predominantly pro-inflammatory effects. Research on this cytokine has contributed to our understanding of immunology and has revealed some of the mechanisms involved in the regulation of cell death and survival. TNFα was initially described as an endogenous factor involved in "killing tumors" when the host was exposed to an infection (mid 1800s)(168). In early 1900s, it was observed that when killed strains of gram-negative bacteria were injected in skin mouse tumors, it caused hemorrhagic necrosis of the tumors. In 1985, Old and colleagues identified a protein in the serum of rabbits treated with lipopolysaccharides (LPS; constituents of bacteria's outer wall) that was responsible for the hemorrhagic necrosis of tumors (169). It was named tumor necrosis factor (TNF), and later named TNFα after the discovery of lymphotoxin or TNF-β. Coincidentally Kawakami and Cerami identified a molecule responsible for the wasting syndrome seen in many diseases, such as chronic infection (170, 171). This molecule was named cachectin, since it was responsible for the induction of cachexia; later, it was found to be identical to TNFα (172).

In 1985, human TNFα was cloned and its amino acid sequence identified (173). The active form of TNFα is a bell-shaped molecule composed of 3 identical subunits, each of ~17 kDa (molecular mass of trimeric is ~51 kDa), similar to the structural motif of viral coat proteins. TNFα is first secreted as a pro-TNFα, which is a transmembrane ~ 26 kDa protein that is cleaved by a membrane-bound metalloproteinase, TNFα converting enzyme (TACE), to generate the mature TNFα. Membrane bound TNFα is also biologically active and may mediate cytotoxicity (173-175).

TNFα can be produced by macrophages and other leukocytes after cytokine or LPS stimulation and plays an important role orchestrating the inflammatory response. TNFα causes endothelial cells and granulocytes to synthesize adhesion molecules that allow the attachment of granulocytes (first phase of the response to injury) to blood vessels and their migration into tissues (176, 177). TNFα is also a potent signal for stimulating granulocytes to produce toxic oxygen free radicals that destroy bacteria, and plays a role in the activation of T- and B-lymphocytes. Furthermore, TNFα stimulates endothelial cells to secrete chemoattractants (178), inflammatory factors, (176, 177, 179) colony-stimulating factors, and induces the release of acute-phase proteins by the liver (IL-6, C-reactive protein) (180). In addition to these pro-inflammatory actions, TNFα is involved in the modulation of haematopoiesis and of short or long-term immunity (reviewed in (181)).

Interestingly, cells of the vascular wall can also produce TNFα. For instance, endothelial cells produce both soluble and membrane-bound forms of TNFα (182). However, the role of endogenous-produced TNFα in endothelial function is unclear.

TNFα effects are mediated by two surface receptors, TNFR1 (p55) and TNFR2 (p75). TNFα receptors are members of a family of homologous receptors termed TNFα receptor superfamily that includes Fas, TRAIL receptors, and RANK (reviewed in (181)). Interestingly, the existence of a 60 kDa mitochondrial receptor for TNFα has also been reported, but its function is unknown (183).

TNFR1 is predominantly localized in the Golgi with lower surface expression compared to TNFR2, which is primarily expressed on the cell surface. The Golgi TNFR1 acts as a pool for replenishing the expression of TNFR1 on the plasma membrane (184, 185). It has been hypothesized that TNFR2 acts as a "ligand passing" to TNFR1 either at the plasma membrane or following TNFR1 endocytosis (185, 186). Moreover, there is also evidence that suggests that TNFR2 primarily responds to membrane-bound forms of TNFα (187, 188).

Most of the pro-inflammatory effects of TNFα are mediated by TNFR1 (Figure 1.5), which leads to the activation of inflammatory transcription factors such as NF-κB or AP-1. For instance, NF-κB activation mediates E-selectin, V-CAM-1 and ICAM-1 expression, whereas TNFα induced-E-selectin expression is mediated by AP-1 activation. Interestingly, NF-κB upregulates the expression of TNFα (189), thus, TNFα can induce its own production, creating an autoregulatory feedback loop.

Binding of TNFα to monomeric receptors results in receptor trimerization, which causes clustering of specific adapter signaling proteins to the intracellular domain (reviewed in (190)). Upon ligand-receptor binding, a complex formed by TNFR associated Death Domain (TRADD), Receptor Interacting Protein (RIP) and TNFR associated factor (TRAF) activates members of the MAP kinase family that mediate the

activation of transcription factors (190). TNFR1 activation can induce apoptosis through the recruitment of death effector adaptor proteins, which results on activation of the caspase cascade (Figure 1.5). On the other hand, TNFR1 through TRAF2 leads to activation of NF-κB and c-Jun NH_2-terminal kinase (JNK) (193) and induces anti-apoptotic genes such as those encoding IAPs and BCL-X (191, 192)(Figure 1.5). Indeed, NF-κB inhibition greatly enhances apoptosis by TNFα. Thus, TNFα activates both pro- and anti-apoptotic signaling pathways

TNFR2 does not have a death dominion and it is linked to anti-apoptotic signaling pathways such IAPs and NF-κB by stimulation of the TRAF2 signaling pathway (194). However, concomitant stimulation of TNFR1 and TNFR2 promotes TNFα-induced apoptosis (195). This is caused by TNFR2 mediated degradation of TRAF2 that is required for the activation of JNK and NF-kB (196). Furthermore, mice lacking TNFR2 have enhanced TNFR1-dependent inflammatory responses (197), suggesting that interactions between both receptors modulate the effects of TNFα.

TNFα is involved in the pathogenesis of atherosclerosis. Indeed, TNFα levels are associated with carotid intima-media thickness, a marker of atherosclerosis (198). Moreover, TNFα induces also a pro-thombotic phenotype by increasing the expression of tissue factor-like procoagulant activity (199), inhibiting the thrombomodulin/protein C anticoagulation pathway (200), and blocking fibrin dissolution by stimulation of the type I inhibitor of plasminogen activator (201). Hence, higher serum levels of TNFα after a myocardial infarction are associated with risk of recurrent coronary atherothrombotic events (202).

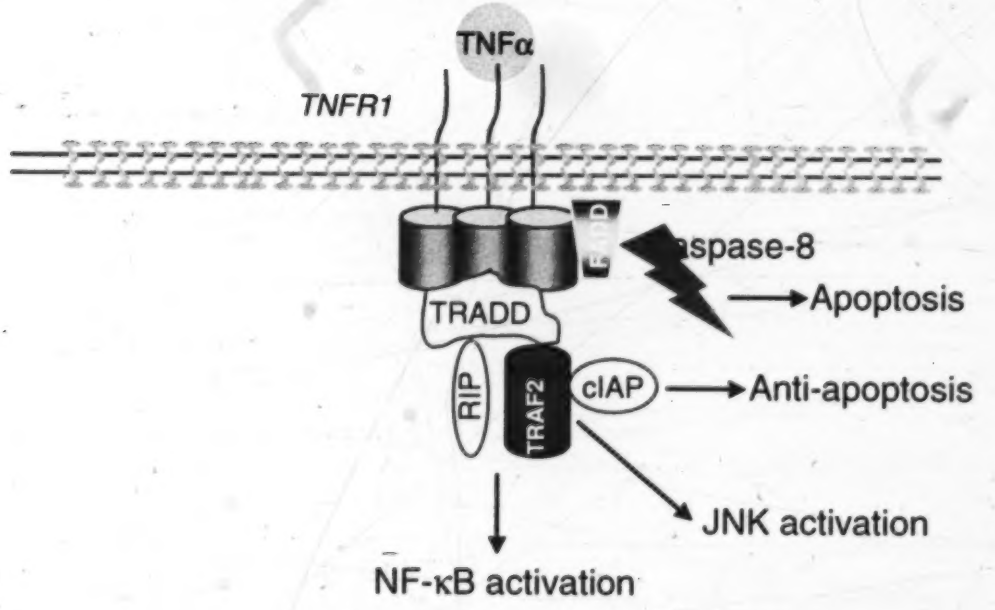

Figure 1.5 Signaling pathways of TNFα via TNFR1.

Binding of TNFα to TNFR1 results in the recruitment of important adaptor proteins TRADD, TRAF2, RIP, and FADD. These adaptor proteins in turn recruit additional key pathway-specific enzymes. Modified from *Chen et al* (193).

TNFα effects on the vasculature can also lead to alterations in vascular tone. Indeed, we recently found that higher levels of TNFα are associated with higher risk of hypertension (203). This action of TNFα may be mediated by altering the balance between vasodilators/vasoconstrictors in the vascular wall. In endothelial cells in culture, TNFα decreases the expression of eNOS by altering mRNA stability (204). Moreover, TNFα is a potent inducer of NAD (P) H oxidase (205, 206), a major source of superoxide anion in the vasculature, which inactivates NO to form peroxynitrite (207). Hence, TNFα can decrease NO availability by both decreasing its production and increasing its inactivation by superoxide anion.

TNFα can also induce the formation of vasoconstrictors such as ANG II. Indeed, TNFα stimulates the expression of Angiotensinogen (208). Moreover, TNFα can increase the expression of AT_1R and promote ANG II effects on collagen deposition in fibroblasts (209). Furthermore, overexpression of TNFα in the heart is associated with activation of the cardiac renin-angiotensin system and alterations in cardiac remodeling (210). Thus, the role of interactions between TNFα and ANG II on vascular and endothelial function is addressed in chapters IV and V of this thesis.

1.5.3 Estrogen deficiency and TNFα

Clinical and experimental observations suggest that estrogen has modulator effects on immune function. In fact, autoimmune diseases occur more frequently in women than in men, and estrogen alters the course of these diseases (211, 212). Moreover, the estrogen receptor α or β knockout mice (213, 214), as well as estrogen deficient mice due to absence of the enzyme aromatase (215), develop severe autoimmune disorders.

There is evidence that suggests that the decline of the ovarian function with menopause is associated with spontaneous increase in levels of TNFα. Pacifici et al showed that oophorectomy in women, increases the spontaneous release of TNFα from monocytes, which was reverted by estrogen replacement (216). Moreover, some clinical studies have reported increase in serum TNFα levels after menopause (217-219). Interestingly, the increase in TNFα levels was independent of other factors such as obesity and fat content (219). These observations suggest that the absence of estrogen may directly contribute to increase TNFα levels in menopausal women.

Indeed, some studies have shown that estrogen has inhibitory effects on TNFα transcription. Estrogen receptors function as ligand-dependent transcription factors that bind to specific consensus regulatory sequences, referred to as estrogen response elements (ERE) located within the regulatory regions of target genes (220). However, TNFα promoter lacks a classical ERE and the repressor effects of estrogen on TNFα, seems to be mediated by interactions of estrogen receptors with other transcription factors such as AP-1 (220).

Srivastava et al (221), found that estrogen downregulates cytokine-induced TNFα gene expression by decreasing the activity of the Jun NH(2)-terminal kinase (JNK), resulting in decreased phosphorylation and nuclear binding of c-Jun/c-Fos and JunD/c-Fos heterodimers to the AP-1 consensus sequence in the TNFα promoter and, thus, to decreased transactivation of the TNFα gene (221). Similarly, An et al (222), found that estrogen inhibited the activation of the TNFα promoter acting on an AP-1-like site of the

TNF-responsive element (TNF-RE) (222). Interestingly, estrogen receptor β was more potent than receptor α at repressing the TNFα promoter (222).

Although these observations suggest that estrogen deficiency is associated with an increase in TNFα levels, whether this increase in TNFα is associated with alterations in vascular function is unknown, and it is the main hypothesis of this thesis.

1.6 SUMMARY AND SPECIFIC HYPOTHESES

TNFα is a cytokine involved in the pathogenesis of vascular disease. The cells of the vascular wall, in particular the endothelium, are very susceptible to the effects of this cytokine. We hypothesized that estrogen deficiency may be associated with increased TNFα levels, which in turn would alter vascular function.

The specific hypotheses and aims for each study of this thesis are as follows:

ARTICLE I: Estrogen deficiency is associated with an increase in TNFα levels, which will affect vascular function by decreasing nitric oxide availability.

Aim 1: To investigate the effects of estrogen deficiency on circulating levels of TNFα in aging female rats.

Aim 2: To determine the effects of estrogen deficiency on NO modulation of vasoconstrictor and endothelial-dependent vasorelaxation of mesenteric arteries.

Aim 3: With the use of a selective inhibitor of TNFα, investigate the effects of role of TNFα inhibition on NO modulation of vascular function in estrogen deficient rats.

ARTICLE II: TNFα inhibition improves vascular relaxation to fluid shear stress in estrogen deficient rats.

Aim 1: To determine the role of NO on vasodilator responses to flow.

Aim 2: To investigate the effects of estrogen deficiency on vascular responses to flow in aging rats.

Aim 3: To determine the role of endogenous TNFα on vascular responses to flow in aged-estrogen deficient rats.

ARTICLE III: TNFα promotes vasoconstriction and vascular dysfunction during estrogen deficiency by promoting the actions of ANG II.

Aim 1: To determine the effect of estrogen deficiency on ANG II modulation of vasoconstriction in mesenteric arteries.

Aim 2: To investigate the effects of estrogen deficiency on the expression of AT_1R and ACE in mesenteric arteries.

Aim 3: To determine the role of TNFα on ANG II modulation of vasoconstriction in mesenteric arteries in aged-estrogen deficient rats.

ARTICLE IV: ANG II can induce the production of TNFα and MMP-2 from endothelial cells, and TNFα mediates the effects of ANG II on MMP-2 release.

Aim 1: To determine the effects of ANG II on MMPs release from endothelial cells.

Aim 2: To investigate whether ANG II induces the production of TNFα in endothelial cells.

Aim 3: To determine whether TNFα mediates the effect of ANG II on MMP-2 release.

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CHAPTER 2. EFFECTS OF ESTROGEN DEFICIENCY ON TNFα LEVELS AND ROLE OF TNFα ON NITRIC OXIDE MODULATION OF VASCULAR FUNCTION DURING ESTROGEN DEFICIENCY.

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2.1 INTRODUCTION

Cardiovascular disease is the leading cause of death for women in developed countries. Although menopause is considered a major risk factor for vascular disease, the pathophysiological mechanisms linking the decrease in ovarian hormones that occurs in menopause with alterations in vascular function are still unclear.

NO is an important vasodilator and has an important role in the control of vascular homeostasis including its ability to modulate the actions of vasoconstrictors such as the α-1 adrenergic agonist phenylephrine (1). In fact, a decrease in NO modulation of vascular function is associated with higher risk of developing vascular disease (2). Animal and human studies have shown that the decline of the ovarian function is associated with decreased NO (3-5). Although the mechanisms remain unknown, the decrease in bioavailable NO seems to be due to both a decrease in its production and to an increase in its inactivation by superoxide anion that reacts with NO to form peroxynitrite.

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine that is proposed to be involved in the pathogenesis of vascular dysfunction. In endothelial cells in culture, TNFα decreases the expression of endothelial nitric oxide synthase (eNOS)(6), which is the primary enzyme involve in NO production in the vasculature (7). Moreover, TNFα

can induce the activity of NAD(P)H oxidase (8, 9), an enzyme that is a major source of superoxide anion in the vasculature (10).

Studies have shown that postmenopausal women have higher TNFα levels compared with pre-menopausal women (11, 12). However, in those studies vascular function was not evaluated and therefore, whether the increase in TNFα levels with estrogen deficiency is associated with changes in vascular function is largely unknown. Thus, we tested whether *in vivo* TNFα inhibition improves vascular function in estrogen deficient rats. The aim of the present study was to investigate whether estrogen deficiency is associated with an increase in circulating levels of TNFα, and to determine the effects of 4 weeks of *in vivo* TNFα inhibition on vascular reactivity. We evaluated the sensitivity of mesenteric arteries to adrenergic constriction and the modulation of this constriction by NO and superoxide anion. Moreover, we investigated changes in endothelial-dependent vasorelaxation, and the effects of TNFα inhibition on vascular expression of eNOS and NAD(P)H oxidase.

2.2 METHODS

2.2.1 Animal model

This study was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and was in accordance with the Canadian Council on Animal Care. Female Sprague-Dawley rats were obtained from Charles River, Canada (Montreal, Quebec) and were housed in the facilities of the University of Alberta until experimentation at 12-15 months of age. This age was chosen since the animals have

attained their state of reproductive senescence (i.e. similar to the postmenopausal state of women), however, rats experience constant estrus at the end of their reproductive age and continue to produce variable levels of estrogen. Thus, to decrease the variability on estrogen levels in these aged animals, we remove the ovaries at the time of the initial treatment. Then, we randomly assigned the animals to different treatments.

2.2.2 Experimental design

To investigate the effects of estrogen deficiency/replacement on circulating levels of TNFα, rats were treated with either a placebo pellet (n=8) or an estrogen pellet (1.5 mg/pellet, 60 day release, Innovative Research of America; n=8), which results in maximal serum estrogen levels (~80 pg/mL) similar to that of intact cycling rats. Moreover, to evaluate the role of TNFα inhibition, rats were treated with either Etanercept (a TNFα inhibitor, Immunex Corporation, Thousand Oaks, CA), subcutaneously administered at 0.3 mg/kg, three times a week (Etan, n=12), or placebo (subcutaneous injection of dd H_2O; Placebo, n=15) for 4 weeks prior to experimentation. Etanercept is composed of the extracellular ligand-binding portion of the human 75 kilodalton (p75) TNFR2. Thus, Etanercept binds and inactivates circulating TNFα. The Etanercept dose for chronic studies was chosen based on effective TNFα inhibition from previous studies in humans and rats (13, 14). Rats were sacrificed by exsanguination while under anesthesia (sodium pentobarbitol, ~60 mg/kg-body weight). A blood sample was taken and serum was obtained by centrifugation. Samples were snap-frozen (–80°C) for subsequent measurement of TNFα levels.

2.2.3 Vessel Preparation

A portion of the mesentery was excised and immersed in ice-cold *N*-(15)piperazine-*N'*-[2-ethanesulfonic acid]-buffered physiological saline solution (HEPES-PSS) which contained the following (in mmol/L): NaCl 142, KCl 4.7, $MgSO_4$ 1.17, Ca_2Cl 1.56, KH_2PO_4 1.18, HEPES 10, and glucose 5.5. Resistance-sized arteries (diameter ~200 µm) were dissected and connected to an isometric myograph system (Kent Scientific Corp.) as previously described (16). Four separate baths were used to study arterial segments simultaneously. Force production was recorded on a data acquisition system (Workbench, Strawberry Tree Inc.).

2.2.4 Vascular Function Studies

Sensitivity of mesenteric arteries to vasoconstriction was evaluated with phenylephrine (PE). Cumulative concentrations of PE (0.1 to 50 µmol/L) were added to the bath and force was measured. After completion of each dose-response curve, a 30-minute recovery period was allowed, during which the baths were changed every 10 minutes with fresh HEPES-PSS. To investigate the modulation of PE constriction by NO and superoxide anion, vessels were preincubated with inhibitors for 15 minutes prior to PE concentration-response curves. PE constriction curves were generated in the absence or presence of the NOS inhibitor nitro-L-arginine methyl ester (L-NAME; 100µmol/L; Calbiochem, CA)(17) and a superoxide scavenger (MnTBAP; 100 µmol/L; Calbiochem, CA) (18). All constriction curves were normalized to 100% for individual vessels.

To evaluate endothelium-dependent and independent vasorelaxation, vessels were pre-constricted with PE to 50% of maximal constriction, and exposed to cumulative

concentrations of bradykinin (0.01 to 1 µmol/L) or sodium nitroprusside (0.001 to 1 µmol/L).

2.2.5 Measurement of TNFα

In order to distinguish bioactive TNFα from that bound to Etanercept (inactive), serum TNFα was measured using the L929-8 bioassay, which allows distinguishing between free (bioactive) or bound forms of TNFα (19). Briefly, L929-8 cells (an isolated subclone of the murine fibroblastoid cell line L929) were cultured in the wells of a 96-well flat-bottomed microtiter plate with medium (IMDM) containing 10% FBS plus 2 µg/mL Actinomycin D (Kindly donated for the laboratory of Dr. Larry Guilbert, University of Alberta) for 2 hours at 37° in 5% CO_2 in air. Fifty microliters of serum or recombinant TNFα standards (1.56 to 200 pg/mL) were then added in triplicate to appropriated wells and incubated at 40°C for 20 hours. Cell viability was assessed by incubation for 2 hours with neutral red dye (0.05% in PBS), which is taken up by vital cells. Then, supernatant vial is removed, the adherent cells are washed with PBS, and color is developed with 0.05 M NaH2P04 in 50% ethanol. The optical density at 570nm of each well, which reflects the number of cells still viable, was measured on an automated microplate reader. The concentration of TNFα in the sample can then be calculated by comparison with a standard curve constructed with the TNFα standards. The lower limit of assay sensitivity is approximately 200 fg/mL of pure, recombinant TNFα. This bioassay also detects TNFβ, but it is not affected by other known cytokines.

2.2.6 Western Blot analysis

Mesenteric arteries were dissected and homogenized in eppendorf tubes (containing a protease inhibitor cocktail to inhibit serine, cysteine and aspartic proteases to prevent degradation; Sigma) using a small tissue homogenizer. The Bradford assay was employed to measure protein concentration. Twenty-five micrograms of protein were loaded onto a SDS-PAGE 9 % gel and transferred to a nitrocellulose membrane. Membranes were then probed either with goat polyclonal anti eNOS antibody (1:1000; Santa Cruz Biotechnology), anti gp91phox (1:100; Santa Cruz Biotechnology), or p22phox (1:100; Santa Cruz Biotechnology). Specificity of primary antibodies was tested in extracts of mesenteric arteries by using specific blocking peptides (Santa Cruz Biotechnology). Primary antibodies were preabsorbed for 30 minutes with a five times higher concentration of specific blocking peptide before probing the membranes. The primary antibody was then detected with a peroxidase-conjugated host specific secondary antibody (1:2000; Santa Cruz Biotechnology). Membranes were scanned with a Fluor Multimager and bands were quantified by densitometric analysis. After initial exposure to these antibodies, membranes were washed three times with 0.1 % TPBS, and then probed with anti α-actin (as a loading control; 1:500; Santa Cruz Biotechnology).

2.2.7 Data analysis

Data from each dose-response curve was fitted to the Hill equation, and a straight line generated by linear least-squares regression analysis. The concentration that would give 50% constriction (EC_{50}) for each individual artery was determined from this line and the mean ± SE calculated from the curves. Tension (T) was calculated using the following formula: T=Force (milliNewtons; mN) / 2X axial length (mm^2). Analysis of

variance (ANOVA) was used for statistical analysis among groups. Post hoc analysis was performed using Tukey's test. A Student's *t* test was used to compare EC_{50} between two groups. Tests were considered significant at $P<0.05$.

2.3 RESULTS

2.3.1 Effects of estrogen deficiency and Etanercept on TNFα levels

Ovariectomy resulted in low serum estrogen levels in animals treated either with placebo (Plac; 13.7 ± 1.7 pg/mL) or Etanercept (Etan; 16 ± 0.9 pg/mL). However, estrogen-replaced animals have serum estradiol levels (63.2 ± 15.2 pg/mL) within the physiological range.

We first investigated the effect of estrogen or chronic TNFα inhibition on serum bioactive TNFα. Estrogen replaced rats had TNFα levels similar to that of intact cycling animals (6.3 ± 4 and 4.4 ± 4 pg/mL, respectively). However, estrogen deficient animals had higher serum bioactive TNFα levels compared to either estrogen replaced or Etanercept treated animals (pg/mL: 31.62 ± 3.9 (Plac) vs. 4.4 ± 4 (Estrogen) and 11.48 ± 3.2 (Etan); Figure 2.1).

2.3.2 Effects of TNFα inhibition on vascular reactivity in estrogen-depleted rats

To further assess the pathophysiological role of TNFα in estrogen-depleted animals, we tested the effects of chronic TNFα inhibition on vascular function. Treatment with Etanercept decreased the sensitivity to PE compared with placebo control animals (Etan vs. Plac; EC_{50}= 4.72 ± 0.7 vs. 2.22 ± 0.1 μmol/L; Figure 2.2A). However, there were no

differences in maximum tension between Etanercept and placebo groups (3.8 ± 0.8 and 3.9 ± 0.7 mN/mm^2, respectively). Incubation with L-NAME resulted in a similar increased in maximum tension in both Etanercept and placebo animals (5.0 ± 0.8 and 5.0 ± 1.1 mN/mm^2 respectively). However, L-NAME significantly increased the sensitivity to PE only in Etanercept treated animals (EC_{50}: without and with L-NAME = 4.72 ± 0.7 and 1.98 ± 0.27 µmol/L; Figure 2.2B) but not in placebo animals (EC_{50}; without and with L-NAME = 2.22 ± 0.1 and 1.83 ± 0.3 µmol/L; Figure 2.2B). These data indicate that TNFα is involved in the decrease of NO modulation of vasoconstriction in estrogen-depleted animals.

The effect of superoxide anion on PE-induced constriction was evaluated by pre-incubating the vessels with a superoxide scavenger (MnTBAP) before the PE-challenge. MnTBAP pretreatment did not affect maximum tension to PE (Etanercept: 4.0 ± 0.8 vs. placebo: 3.7 ± 0.7 mN/mm^2). However, MnTBAP decreased the sensitivity to PE in mesenteric arteries from placebo animals (EC_{50}: without and with MnTBAP= 2.22 ± 0.1 and 4.85 ± 0.5 µmol/L respectively; Figure 2.3) but not in Etanercept treated animals (EC_{50}: without and with MnTBAP = 4.72 ± 0.7 vs. 3.86 ± 0.3 µmol/L; Figure 2.3).

Similar findings were obtained in a subset of vessels exposed to the soluble polyethylene glycol–superoxide dismutase; PEG–SOD (EC_{50}: Etan and Plac = 3.6 ± 0.8 and 4.77 ± 0.4 µmol/L). Since superoxide anion scavenging only affected the sensitivity of vessels from placebo animals, we tested whether the decreased sensitivity was due to NO. A subset of vessels from animals treated with placebo was co-incubated with MnTBAP and L-NAME. In the presence of MnTBAP, L-NAME enhanced PE

constriction (EC_{50} = 4.85 ± 0.5 (MnTBAP) and 2.70 ± 0.7 (MnTBAP + L-NAME) µmol/L; Figure 2.4), indicating an increase on NO modulation in the vessels of placebo animals in the presence of a superoxide scavenger.

To further determine differences in NO modulation, we evaluated endothelial-dependent vasorelaxation with bradykinin. Relaxation was increased in vessels from Etanercept treated animals compared with controls (% maximal relaxation; Etan vs. Plac = 89 +/- 6 vs. 65 +/- 7; $P<0.05$; Figure 2.5A). However, there were no differences in endothelial-independent vasodilation using sodium nitroprusside (% maximal relaxation; Etan vs. Plac = 93 +/- 3 vs. 92 +/- 2; Figure 2.5B).

2.3.3 Effects of TNFα inhibition on vascular expression of eNOS and NAD(P)H oxidase

To evaluate whether changes in function were accompanied by alterations in tissue protein levels, we evaluated the expression of eNOS and NAD(P)H by Western blot. eNOS expression in animals treated with Etanercept was increased compared with placebo (Figure 2.6A). In contrast, the expression of NAD(P)H oxidase p22phox and gp91phox was decreased by TNFα inhibition (Figures 2.6B and C).

2.4 DISCUSSION

TNFα levels have been shown to be higher in postmenopausal women compared with premenopausal women (11, 12). However, in those studies vascular function was not evaluated, thus, whether this increase in TNFα levels is associated with vascular dysfunction is unknown. In the present study, estrogen depleted animals had a two-fold

increase in serum TNFα levels compared with estrogen-replaced animals. Moreover, elevated TNFα levels impair vascular function in estrogen-depleted animals as evidenced by the chronic TNFα inhibition studies.

The vascular endothelium is particularly affected by the actions of TNFα. After cytokine stimulation endothelial cells undergo morphological alterations that result in a pro-thrombotic and pro-inflammatory phenotype (activation/dysfunction). However, a common initial change for these endothelial alterations is a decrease in NO availability (2, 20). TNFα decreases eNOS expression in endothelial cells (6) and can also increase the release of superoxide anion that inactivates NO (21). These effects can lead to decreased NO bioavailability. Hence, *in vitro* administration of TNFα has been shown to reduce endothelium-dependent vasorelaxation (22). However, whether increases in *in vivo* TNFα levels in a model of menopause are associated with vascular dysfunction was the focus of the present study.

In this study, TNFα inhibition with Etanercept was associated with a decrease in serum TNFα in estrogen deficient animals. Interestingly, the reduction in TNFα by this inhibitor was accompanied by an almost three-fold increase in the tissue expression of eNOS and an increase in maximum endothelium-dependent vasorelaxation to bradykinin without changes in smooth muscle sensitivity to NO as evidenced by no changes in sensitivity to the NO donor sodium nitroprusside. These observations strongly suggest that circulating TNFα can target vascular eNOS leading to a decrease in NO formation. Nitric oxide modulates the vascular contraction to alpha adrenergic agonists (23, 24). Moreover, increased NO scavenging by superoxide anion has been shown to increase the constrictor responses to PE.(25) On the other hand, superoxide anion could promote

vasoconstriction by facilitating the mobilization of cytosolic Ca^{2+} in vascular smooth muscle cells and/or by causing Ca^{2+} sensitization of contractile elements (26). In the present study, treatment with Etanercept resulted in decreased sensitivity (increased EC_{50}) to PE, without affecting maximum contraction. Thus, this suggests that the treatment has modified the endothelial modulation of vasoconstriction rather than having a direct effect on smooth muscle cell contractility.

There was also an absence of NO modulation of PE constriction in estrogen-depleted rats, which was restored in Etanercept treated animals. Moreover, MnTBAP at concentrations found to reduce the biological effects of superoxide anion in several models of oxidative stress (18), decreased PE constriction in placebo animals and restored in part the modulation by L-NAME of PE constriction. Similar results were obtained using an enzymatic superoxide scavenger (PEG–SOD). These observations suggest that superoxide was involved in the scavenging of NO in these animals. An important source of superoxide anion in vascular cells is NAD(P)H oxidase (10). This enzyme can be upregulated by TNFα. TNFα activation of NAD(P)H oxidase in vascular cells is associated with an increase in the expression of the membrane bound component p22phox (8, 9). In this study, placebo rats have increased tissue expression of p22phox and gp91phox subunits compared to animals treated with Etanercept, suggesting that TNFα could mediate the upregulation of NAD(P)H oxidase, which could have contributed to increase the levels of superoxide anion in estrogen-deficient animals.

In conclusion, the decrease in NO modulation seen in estrogen-depleted rats could be due to a decrease in eNOS expression and/or to an increase in NO scavenging by free radicals such as superoxide anion. Some studies have shown that estrogen deficiency is

associated with decreased NO availability and increased superoxide anion in the vasculature of postmenopausal women and ovariectomized animals (3, 27, 28). However, most of these studies have focused on the direct effects of estrogen on free radical production, and little is known about the role of other potential factors that could mediate these alterations in NO availability.

In this study, we are showing that estrogen deficient rats have higher serum bioactive TNFα levels compared with estrogen-replaced animals. Moreover, decrease of bioactive levels of TNFα by a soluble TNFα receptor resulted in increased modulation of vascular function by NO, higher expression of eNOS and decreased expression of NAD(P)H oxidase in mesenteric arteries. These observations suggest that TNFα could be a mediator of vascular dysfunction associated with estrogen deficiency.

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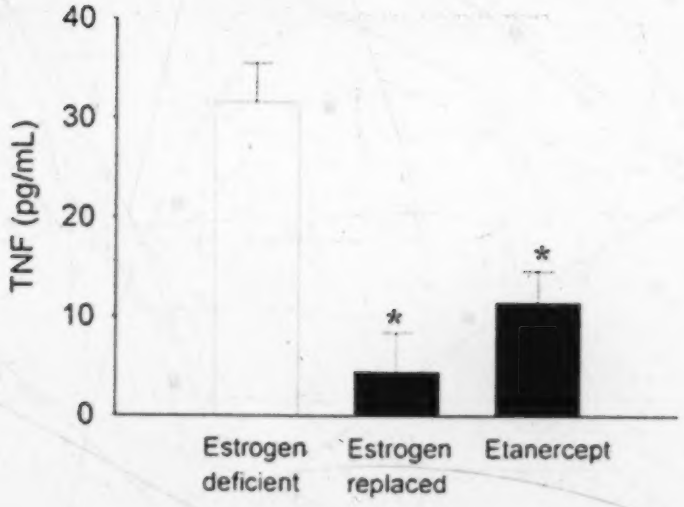

Figure 2.1 Estrogen deficiency is associated with higher serum bioactive TNFα levels, which are reduced by estrogen replacement or chronic TNFα inhibition.

Serum levels of bioactive TNFα in ovariectomized rats treated for 4 weeks either with placebo, 17β estradiol or Etanercept (a TNFα inhibitor). TNFα was quantified using the L929-8 bioassay by comparison to a pure, recombinant TNFα. Individual samples were tested in triplicate. Results represent means ± SD. * $P < 0.05$ versus estrogen deficient animals.

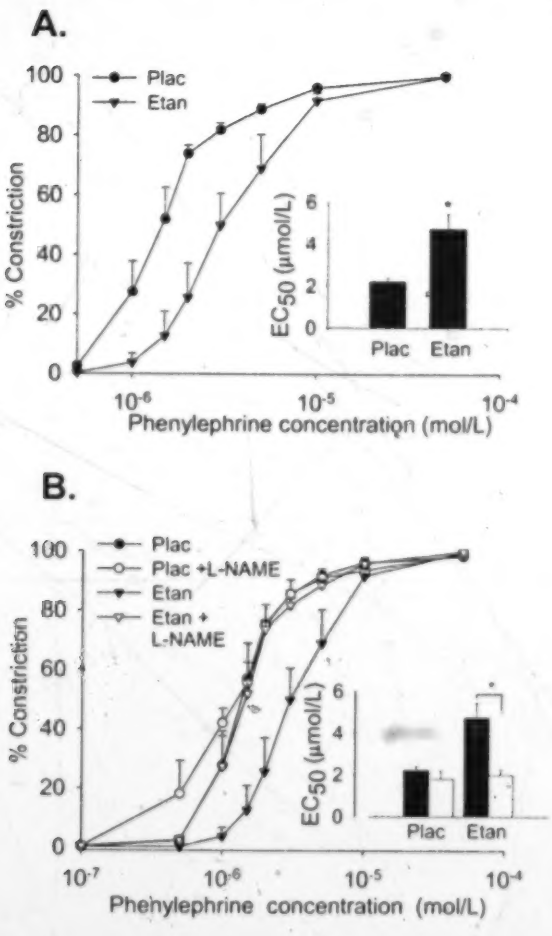

Figure 2.2 Chronic TNFα inhibition increases NO modulation of PE vasoconstriction in estrogen deficient rats.

Panel A: Phenylephrine (PE) concentration response curves and EC_{50} bar graphs of mesenteric arteries from ovariectomized rats treated either with Etanercept (n=15) or control animals (placebo; n=12). Bars represent mean + SE. * $P < 0.05$.

Panel B: PE concentration response curves and EC_{50} in the absence (solid symbols and bars) or in the presence (open symbols and bars) of *in vitro* L-NAME (100 µmol/L; n=8). Circles represent vessels from placebo treated animals. Triangles represent vessels from animas treated with Etanercept. Bars represent mean + SE * $P < 0.05$.

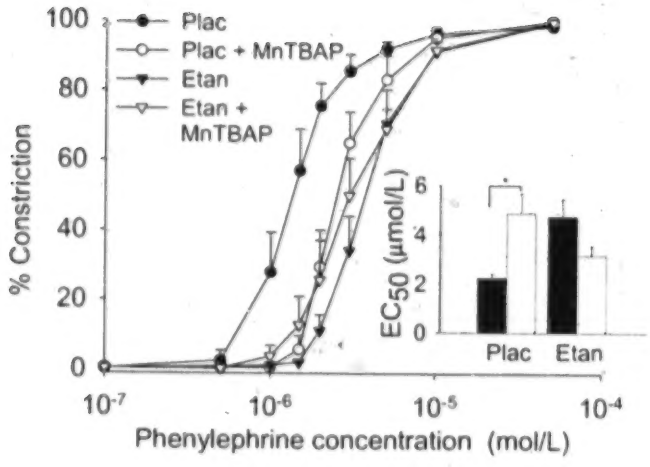

Figure 2.3 Superoxide scavenging decreases the sensitivity to PE vasoconstriction in estrogen deficient rats.

Phenylephrine (PE) concentration response curves and EC_{50} bar graphs of mesenteric arteries from ovariectomized rats treated either with Etanercept (n=15) or control animals (placebo; n=12), in the absence (solid symbols and bars) or in the presence of *in vitro* MnTBAP (10 µmol/L; open symbols and bars; n=6). Circles represent vessels from placebo treated animals. Triangles represent vessels from animals treated with Etanercept. Bars represent mean + SE. * P<0.01 vs. PE alone.

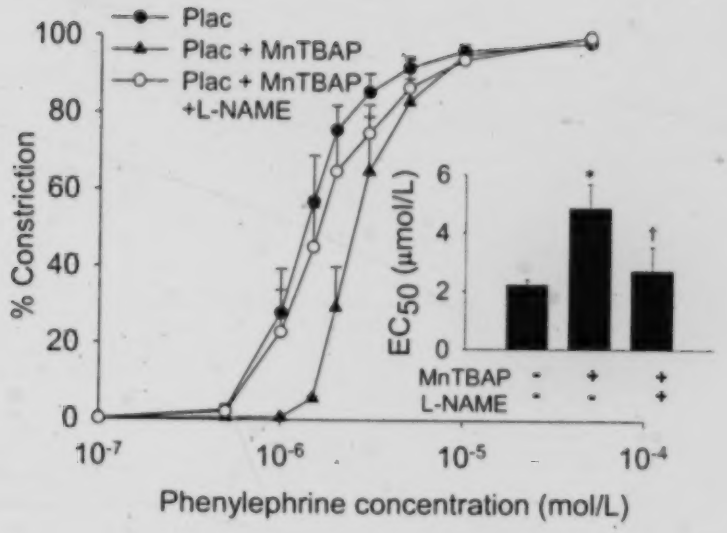

Figure 2.4 Superoxide scavenging increases NO modulation of PE vasoconstriction in mesenteric arteries from estrogen deficient rats.

Phenylephrine (PE) concentration response curves and EC_{50} bar graphs from mesenteric arteries from ovariectomized rats treated with placebo. PE in the absence (n=12) or in the presence of *in vitro* MnTBAP (10 µmol/L; n=6) or L-NAME (100 µmol/L; n=8) or MnTBAP plus L-NAME (n=3). Bars represent mean + SE. * $P<0.05$ vs. Placebo. † $P<0.05$ vs. MnTBAP plus L-NAME.

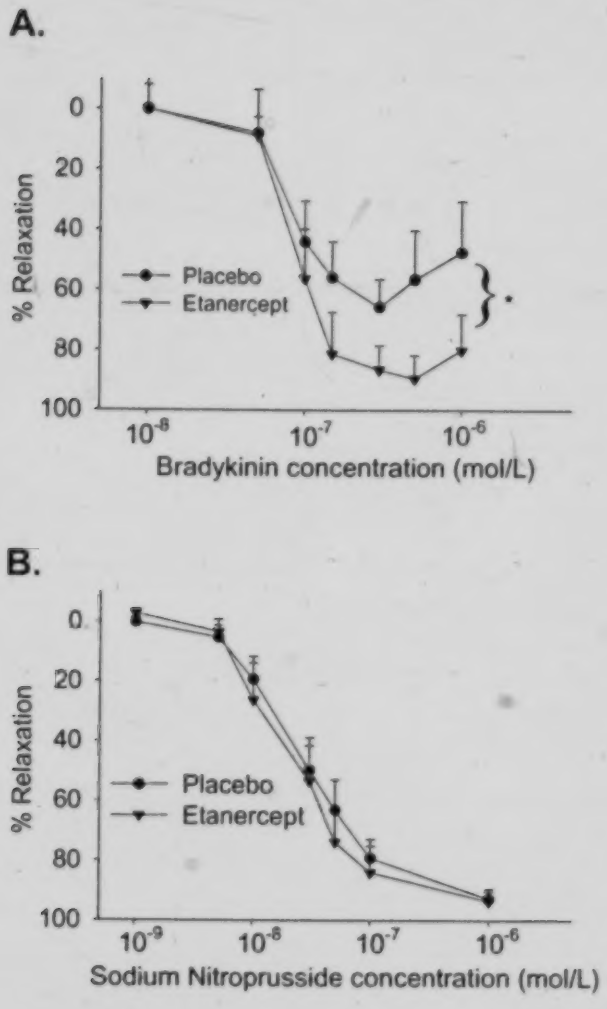

Figure 2.5 Chronic TNFα inhibition increases endothelial-dependent relaxation of mesenteric arteries from estrogen deficient animals.

Panel A: Endothelium-dependent relaxation to bradykinin of mesenteric arteries from ovariectomized rats treated for 4 weeks either with Etanercept (n= 6) or placebo (n=6). Panel B: Endothelium-independent relaxation to sodium nitroprusside of mesenteric arteries from ovariectomized rats treated for 4 weeks either with Etanercept (n= 6) or placebo (n=6). Circles represent vessels from placebo treated animals. Triangles represent vessels from animals treated with Etanercept. * $P<0.05$.

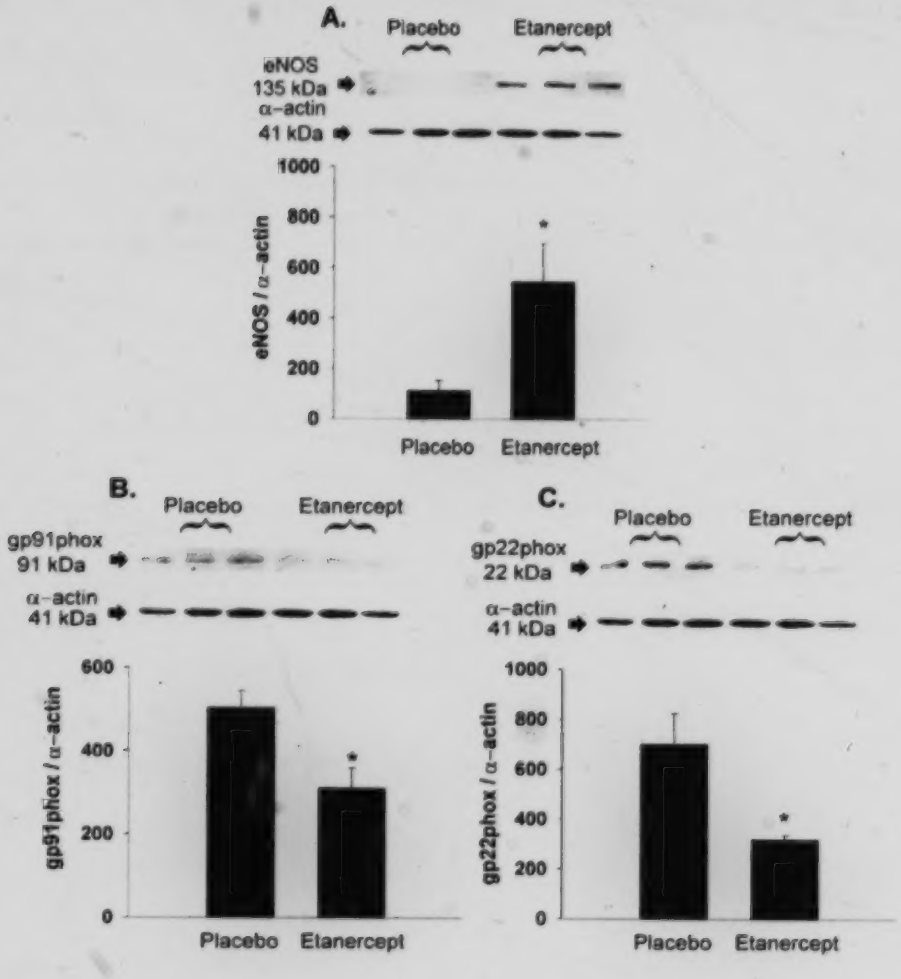

Figure 2.6 Chronic TNFα inhibition increases eNOS and reduces NAD(P)H oxidase expression in arteries from estrogen deficient rats.

Tissue expression of endothelial nitric oxide synthase (eNOS) and NAD(P)H oxidase on mesenteric arteries of ovariectomized rats treated for 4 weeks with Etanercept or control animals. Representative Western blot and summary of densitometric analysis for eNOS (Panel A), NAD(P)H gp91phox (Panel B) or NAD(P)H p22phox subunits (Panel C). Bars represent mean + SD. * $P<0.05$ vs. placebo.

CHAPTER 3. IMPAIRMENT IN VASORELAXATION TO FLUID SHEAR STRESS IN RESISTANCE ARTERIES FROM ESTROGEN DEFICIENT FEMALE RATS IS IMPROVED BY TNFα ANTAGONISM.

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3.1 INTRODUCTION

Aging is associated with alterations in vascular function and higher risk of cardiovascular disease. In women, age-associated alterations in vascular function are related to the onset of menopause (1, 2). Inflammatory factors are involved in the pathogenesis of vascular disorders, and some evidence suggests that cytokines may mediate some of the vascular changes associated with aging. Indeed, we previously found that in a model of aging in female rats, tumor necrosis factor alpha (TNFα), a pro-inflammatory cytokine, is involved in the alterations of vascular function by reducing nitric oxide (NO) availability (3).

Shear stress on the vascular wall is an important mechanism that regulates vascular homeostasis, including vascular tone (4). For instance, in isolated resistance arteries wall shear stress elicited by intraluminal flow can induce vasodilation of constricted arteries (5). Flow-dependent vasodilation is mediated by endothelium-derived factors such as nitric oxide (NO) (6). In fact, NO production by endothelial NO synthase (eNOS) is stimulated by fluid shear stress (7, 8).

Aging has been associated with altered responses to shear stress (9, 10), and although the mechanisms remain unclear, it has been hypothesized that a reduction in NO

availability may mediate these alterations (9, 10). NO modulates vascular levels of shear stress by inducing arterial dilation (4). Thus, reduction in NO availability is associated with either a decrease in vasodilation to flow or constriction (11). This could lead to an increase in the levels of shear stress that may cause endothelial damage and increase peripheral resistance. Accordingly, flow-induced vasorelaxation is decreased in hypertensive disorders (12, 13). Moreover, a decrease in flow-dependent relaxation is an independent predictor of future cardiovascular events (14).

Inflammatory factors such as TNFα play an important role in the pathogenesis of vascular disease (15). In women, aging is associated with an increase in TNFα levels (16), which may reduce NO availability (3). Thus we investigated the effect of aging on vascular responses to shear stress and evaluated the role of selective TNFα inhibition on flow vasodilation in aged female animals. We hypothesized that aged vessels are less sensitive to the vasodilator effects of shear stress caused by a decrease in NO availability, and that these alterations could be improved by TNFα inhibition.

3.2 METHODS

3.2.1 Animal model

This study was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and was in accordance with the Canadian Council on Animal Care. Female Sprague-Dawley rats were obtained from Charles River, Canada (Montreal, Quebec) and were housed in the facilities of the University of Alberta until experimentation at 12-15 months of age. We removed the ovaries at the time of the

initial treatment and randomly assigned the aged animals to different treatments as described in chapter 2.

3.2.2 Experimental design

To investigate the effects of aging on vascular function intact cycling animals (4 months old; proestrus) were used as a control (n= 6). Moreover, to evaluate the role of TNFα, aged rats were treated with either Etanercept (a TNFα inhibitor, Immunex Corporation, Thousand Oaks, CA), subcutaneously administered at 0.3 mg/kg, three times a week (Etan, n=9), or placebo (subcutaneous injection of dd H_2O; Placebo, n=12) for 4 weeks prior to experimentation as previously described. Rats were sacrificed by exsanguination while under anesthesia (sodium pentobarbitol, ~60 mg/kg-body weight).

3.2.3 Vessel Preparation

Vessels were dissected as previously described and transferred to a dual-chamber pressure myograph (Living Systems Instrumentation, Burlington, Vermont). The chamber contains a pair of glass micropipettes filled with HEPES-PSS at room temperature. After the vessel was mounted on the proximal pipette and secured with sutures, it was gently flushed with physiological buffer (10 μL/min) to clear blood from the lumen. Next, the other end of the vessel was mounted in the distal pipette. After mounting, all arteries were examined for ability to maintain pressure. Any decrease in pressure indicated a leak and a new artery was dissected. The second-order mesenteric arteries were equilibrated in warm HEPES-PSS for 30 minutes at an intraluminal pressure of 60 mmHg at zero flow.

3.2.4 Vascular Function Studies

Vessel integrity was evaluated by constriction to a single bolus of phenylephrine (0.1 µmol/L) and relaxation to methacholine (1 µmol/L) to test endothelial dependent relaxation. After the equilibration period vessels were preconstricted with phenylephrine ~ 70 % of their initial internal diameter. Then, flow-diameter relationship was establishing by step increases in flow from 0 to 150 µL/min. Flow was established at a constant intravascular pressure (60 mmHg) by controlling proximal and distal pressures to keep midpoint luminal pressure constant. Intraluminal pressures were controlled in a two-chamber arteriograph through two servo-controlled peristaltic pumps connected to the cannula via a pressure transducer. A digital filar eyepiece (Lasico, Los Angeles, CA) mounted on a compound microscope was used to measure arterial lumen diameters as previously described (27).

Flow rate was calibrated by a Harvard perfusion pump in the range to 0 to 200 µL/min. Each flow step was maintained for ~ 120 seconds before the diameter of the arterioles was measured. After control flow-diameter curves were obtained, vessels were pre-incubated with the NOS inhibitor nitro-L-arginine methyl ester (L-NAME; 100 µmol/L; Calbiochem, CA).(28) Then, after 30 minutes of incubation, changes in diameter in response to step increases in flow were reassessed. At the end of each experiment, diameter measurements were conducted in the absence of extracellular calcium to determine artery passive diameter.

The role of endothelium on flow responses was evaluated by mechanically removal of the endothelium by bubbling air through the vessels for 10 minutes. Confirmation of complete endothelium removal was assessed pharmacologically with a bolus dose of 1

μmol/L methacholine. Sodium nitroprusside (1 μmol/L) was used to evaluate viability in vessels that not responded to methacholine. Percentage of relaxation was calculated after any flow step by using the following formula: % Relaxation = 100 * $(D_1 - D_2)/D_3-D_2)$, where D_1 is the internal diameter in Ca^{2+}-free medium diameter, D_2 is the internal diameter after pre-constriction with Phenylephrine, and D_3 is the internal diameter at any flow step. Shear stress was calculated according to the following equation: Shear stress (Dyne/cm^2) = $4\eta Q/\pi r^3$, where η is viscosity of the perfusate (PSS = ~0.007 poise at 37°C), Q is flow (μL/min) and r is radius (cm) (29).

3.2.5 Data analysis

Data are presented as a mean ± SE. Two-way repeated-measures ANOVA was used to assess differences in vasodilator responses to flow steps between groups, and to test the effect of L-NAME. Student's t-test was used to test differences in maximal dilation between two groups. Tests were considered significant at $P<0.05$.

3.3 RESULTS

3.3.1 Effects of aging and TNFα inhibition on vascular responses to shear stress.

Passive arterial diameters at 60 mmHg were not significantly different between young and aged animals (288 ± 21 vs. 301 ± 18 μms). Compared with young animals, aged animals have a blunted vasodilator response to flow (Figure 3.1A; $P<0.05$). Percentage of maximal dilation was 24 ± 15 and 52 ± 4 in aged and young vessels respectively (Figure 3.1B; $P< 0.05$). Flow rates (0 to 150 μL/min) corresponded to levels of shear stress

ranging from 0 to 45.6 ± 13.2 dynes/cm^2 in young animals and from 0 to 265 ± 81 dynes/cm^2 in aged animals (Figure 3.2; P< 0.05). Maximal relaxation was achieved at higher levels of shear stress in aged animals compared with young (70 ± 31 and 19 ± 6 dynes/cm^2; P< 0.05).

TNFα inhibition with Etanercept improved flow vasodilation in aged animals (Figure 3.3A). Maximal dilation was greater in Etanercept treated animals compared with aged animals treated with placebo (65 ± 15 and 22 ± 13 % respectively; Figure 3.3B; P< 0.05). Levels of shear stress in vessels from Etanercept treated animals were reduced compared with aged animals treated with placebo (Figure 3.4; P<0.05). Passive arterial diameter did not differ between aged groups (Placebo and Etanercept: 288 ± 14 and 314 ± 20 μm).

3.3.2 Effects of endothelial removal and NOS inhibition on flow-mediated vasodilation.

Endothelium denuded vessels did not dilate to flow and showed vasoconstriction (data not shown). NOS inhibition with L-NAME abolished the vasodilation of vessels from young (Figure 3.5A) and aged animals (Figures 3.5B and 3.5C). Indeed, differences in vasodilation to flow among groups were not longer significant after L-NAME pre-treatment (P=0.3). However, the net effect of L-NAME on maximal vasodilation was greater in young animals and aged animals treated with Etanercept compared with aged-placebo animals (Delta change in Max. Relaxation (Etan vs. Placebo): 55 ± 14 vs. 18 ± 7; P< 0.05).

3.4 DISCUSSION

The primary findings of this work are that aging is associated with a decrease in vascular dilation to intraluminal flow and higher levels of shear stress in isolated resistance arteries of female rats due in part to a reduction in endothelial NO modulation. Moreover, TNFα antagonism with Etanercept increased NO mediated vasodilation to flow and decreased levels of shear stress in aged animals.

Shear stress is an important mechanism that contributes to vascular homeostasis. Experimental evidence suggests that shear stress is able to modulate the expression of endothelial genes by the activation of transcription factors (30), and modulates vascular homeostatic processes such as remodelling (31), inflammation (32), and vascular tone. Shear stress regulates the expression of eNOS (33), and during physiological conditions maintains the basal release of endothelial NO (12, 34), which opposes several vasoconstrictor stimuli including neurohormonal stimulation. Shear stress-induced NO release is mediated by acute phosphorylation of eNOS by protein kinases (7, 35). However, in pathological conditions a decrease in NO availabili[illegible]s in chronic vasoconstriction and higher levels of shear stress (12).

In the present study, vasodilation to flow was abolished by either NOS inhibition with L-NAME or endothelial removal. Moreover, after L-NAME incubation, the decrease in vasodilation was significantly larger in young rats compared with aged-placebo animals, suggesting that the greater flow/shear stress-induced dilation of vessels of young female rats is likely to be due to a greater release of NO. These results are consistent with previous findings suggesting that endothelial NO is a primary mediator of flow-dependent relaxation in mesen[illegible]ric arteries (36, 37), and other studies reporting that

vasodilation is reduced with aging (9, 10). However, most of these studies have been conducted in male animals, and only few have demonstrated these alterations in a model of aging in female animals (10).

Moreover, our findings agree with clinical studies showing that flow-dilation of the brachial artery is decreased in postmenopausal women (38). Although these abnormalities have been attributed to higher levels of oxidative stress and a decrease in NO availability, the mechanisms that trigger these changes remain unclear. TNFα is a pro-inflammatory cytokine involved in the pathogenesis of vascular disorders. TNFα reduces NO availability by decreasing eNOS expression (39) or by increasing NO inactivation by superoxide anion through stimulation of NAD(P)H oxidase (40). TNFα formation is regulated by estrogen (41), and the decline of the ovarian function with menopause is associated with spontaneous increases in TNFα levels. In agreement with our hypothesis, TNFα inhibition with Etanercept improved vasorelaxation to flow in aged animals, and increased the modulation by L-NAME suggesting that the increase in vasodilation observed in aged animals treated with Etanercept is mediated by enhancing NO availability.

Regulation of wall shear stress contributes to vascular resistance. Although moderate levels of shear stress are vasculoprotective, higher levels of shear stress may cause endothelial damage and increase peripheral resistance, which are features of hypertensive disorders. In this study, maximal relaxation in young vessels occurred at lower levels of shear stress compared with aged animals. Moreover, vessels from aged animals have an increase in wall shear stress when compared with young animals subjected to similar flow rates. These observations may suggest that aged vessels are less sensitive to shear

stress-induced vasodilation. Interestingly, improvement in NO modulation in aged vessels with Etanercept resulted in a reduction of shear stress levels. However, the levels of shear stress at which maximal dilation occurred did not differ between aged groups, suggesting that there are differences in signaling for NO release between aged and young animals.

The mechanisms by which flow is translated into cellular signaling are still unclear. It has been postulated the presence of a mechanotransducer within the cell membrane, which would trigger an enzymatic cascade (42). Moreover, it has also been proposed that shear stress signaling is mediated by components of the cytoskeleton such as the integrins (43). Therefore, alterations in signaling mechanisms or structural changes in aged vessels could be involved on determining the point of stress needed for maximal relaxation.

In summary, we have shown that in female rats, NO is the primary mediator of flow-dependent relaxation in isolated mesenteric arteries. Moreover, aging is associated with a reduction in shear stress-induced vasodilation as well as in maximal relaxation, which was improved by TNFα antagonism. This effect was related to an increase in NO modulation of vasorelaxation. Taken together, these findings suggest that upregulation of TNFα levels with aging is associated with a decrease in flow-mediated vasodilation leading to higher levels of shear stress that could result in vascular dysfunction.

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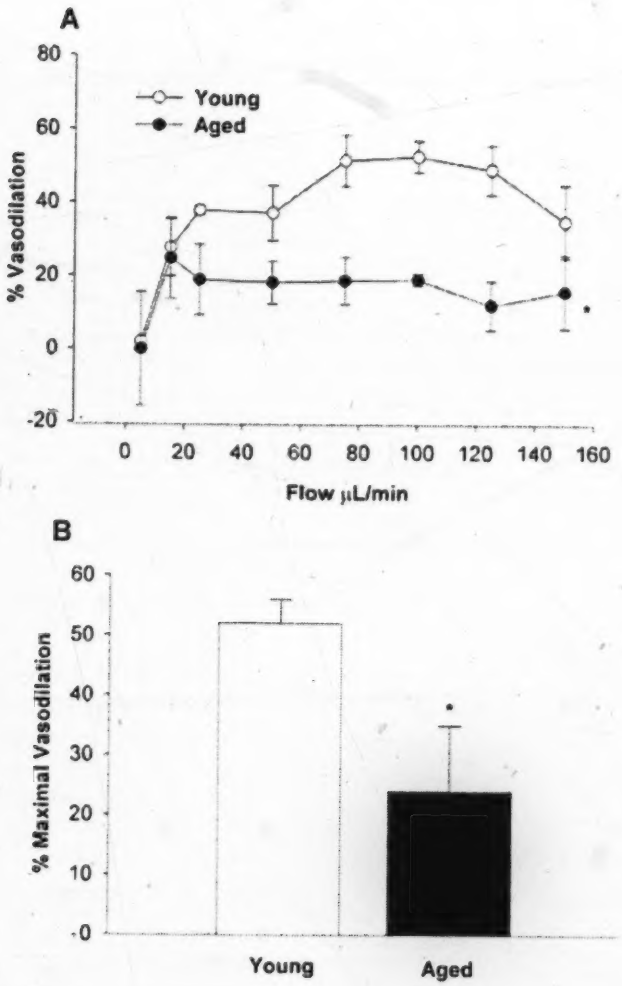

Figure 3.1 Aging is associated with a decrease in flow-mediated vasodilation.

Effects of aging in female rats on flow-mediated vasodilation of mesenteric arteries. Panel A: Flow responses of mesenteric arteries. Open circles represent vessels from young animals (n=6) and solid circles represent vessels from aged animals (n=9). Panel B: Percentage of maximal vasodilation. Bars represent mean ± SE. * $P<0.05$ vs young.

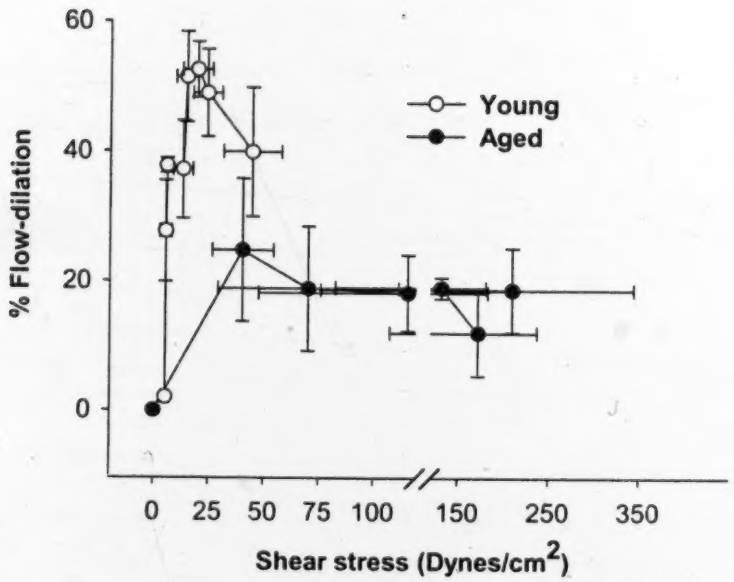

Figure 3.2 Flow results in higher levels of shear stress in vessels from aged rats compared with from young animals.

Shear stress levels and vasodilation to flow of mesenteric arteries from young (open circles; n=9) or aged animals (solid circles; n=9). Horizontal bars represent shear stress (mean ± SE), vertical bars represent percentage of flow vasodilation.

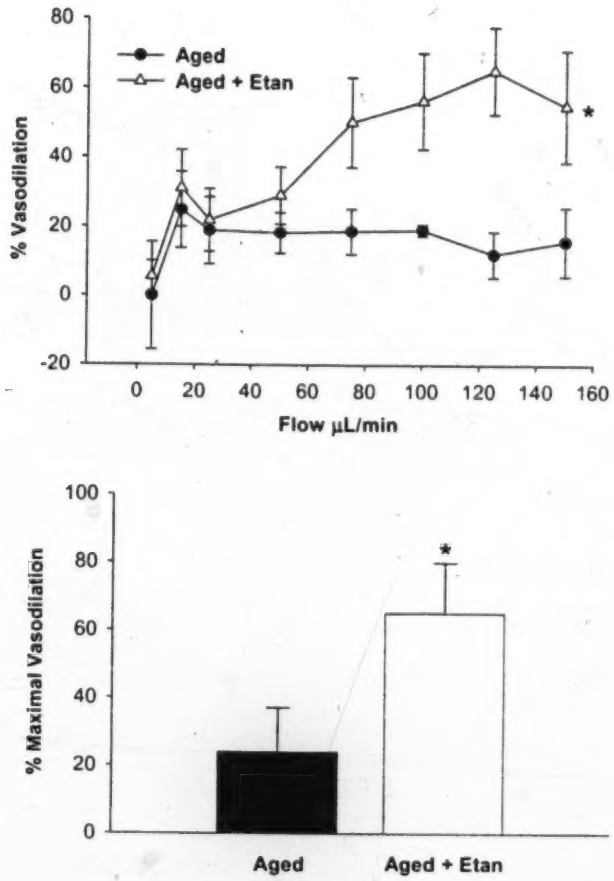

Figure 3.3 TNFα inhibition improves flow-mediated vasodilation in vessels from aged ovariectomized animals.

Effect of *in vivo* TNFα inhibition with Etanercept on flow-mediated vasodilation in aged rats. Panel A: Flow responses of mesenteric arteries. Panel B: Percentage of maximal vasodilation. Circles represent vessels from placebo treated animals (n=9). Triangles represent vessels from animals treated with Etanercept (n=9). Bars represent mean ± SE. * $P<0.05$ vs. Aged placebo animals.

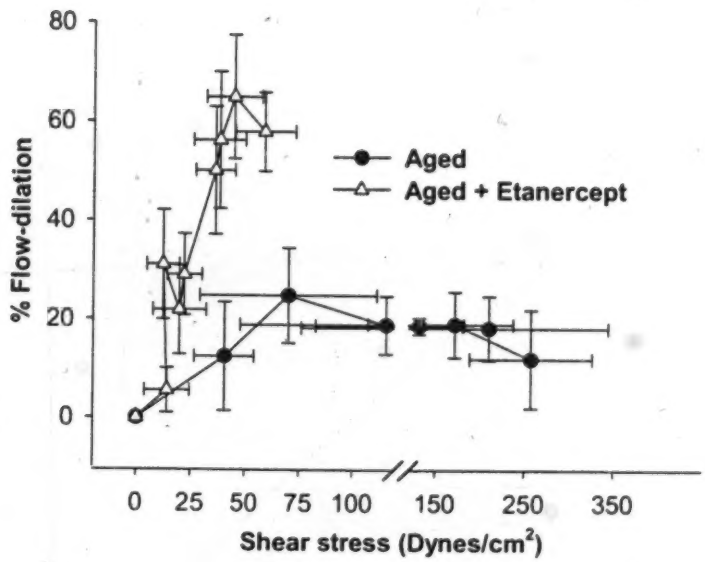

Figure 3.4 Treatment with Etanercept improves vasodilation to flow and decreases shear stress.

Shear stress levels and vasodilation to flow of mesenteric arteries from aged rats treated either with placebo (open circles; n=9) or Etanercept (solid circles; n=9). Horizontal bars represent shear stress (mean ± SE), vertical bars represent percentage of flow vasodilation.

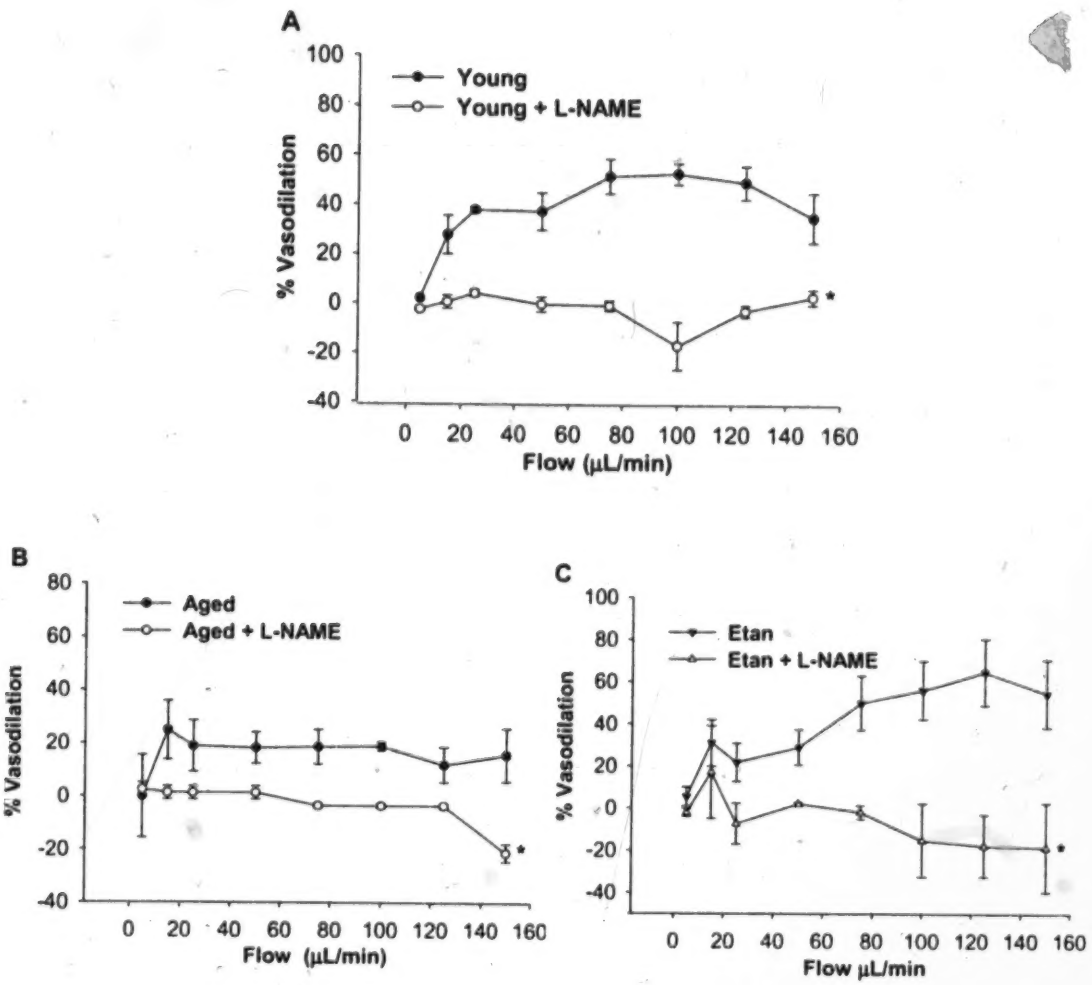

Figure 3.5 NOS inhibition with L-NAME abolishes flow-mediated vasodilation in mesenteric arteries from young and aged animals.

Effects of NOS inhibition on flow-mediated vasodilation. Flow responses of mesenteric arteries from young animals (Panel A; n= 6) or aged rats treated either with placebo (Panel B; n=9) or Etanercept (Panel C; n=9) in the absence (solid symbols) or in the presence (open symbols) of *in vitro* L-NAME (100 µmol/L; n=3). * $P < 0.05$ vs flow alone.

CHAPTER 4. TNF α INHIBITION REDUCES ANGIOTENSIN II MODULATION OF VASOCONSTRICTION IN ESTROGEN DEFICIENT RATS.

A version of this chapter has been submitted for publication to Cardiovascular Research. Arenas et al, 2005.

4.1 INTRODUCTION

Cardiovascular disease is more prevalent in post-menopausal women compared with pre-menopausal women. Mounting evidence indicates that inflammatory factors are involved in the pathogenesis of cardiovascular disease. In previous studies we have found that estrogen deficiency is associated with an increase in circulating levels of Tumor necrosis factor alpha (TNFα), a pro-inflammatory cytokine, which results in alterations of vascular function (1).

A balance between vasodilators and vasoconstrictors modulates vascular tone. Estrogen deficiency has been associated with an increase in endogenous (produced within the vascular wall) vasoconstrictors (2). Angiotensin II (ANG II) is a vasoconstrictor that has been associated with the pathogenesis of atherosclerosis and many vascular disorders (3). Evidence indicates that endogenous ANG II, which can be locally formed in vascular tissues by the action of the Angiotensin Converting enzyme (ACE), plays an important role in the control of vascular tone (4, 5). For instance, endogenous ANG II enhances adrenergic vasoconstriction through activation of ANG II type 1 receptor (AT_1R) (6, 7). Some studies have suggested that estrogen deficiency is associated with alterations in the Renin-Angiotensin System (RAS), and that ANG II is involved in the mechanisms that lead to vascular dysfunction after menopause (8-10). However, few studies have

attempted to investigate the role of these alterations in the function of small arteries. Moreover, the factor(s) that modulate(s) vascular RAS expression during estrogen deficiency remain also unclear.

Experimental evidence indicates that inflammation may alter the formation of ANG II and the expression of its receptors on vascular cells. For instance, inflammatory cytokines can increase the formation of Angiotensinogen (11), as well as the expression of AT_1R in vascular cells (12). Furthermore, RAS inhibition has been associated with anti-inflammatory changes in several models of inflammation, suggesting that chronic inflammation may "activate" RAS, and promote the effects of ANG II, which in turn may induce inflammation. Estrogen deficiency is associated with an increase in TNFα levels (1, 13, 14). However, whether TNFα-ANG II interactions modulate vascular function during estrogen deficiency is unknown.

We hypothesized that in the state of estrogen deficiency, TNFα may be a mediator of the alterations of vascular RAS, promoting the effects of ANG II. This may occur by increasing the local availability of ANG II or/and by upregulating the expression of AT_1R. In this study, we evaluated the effect of estrogen replacement or TNFα inhibition in the vascular expression of ACE, ANG II and AT_1R, and investigated the role of ANG II modulation of vasoconstriction in mesenteric arteries from estrogen deficient rats.

4.2 METHODS

4.2.1 Animal model

This study was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee and was in accordance with the Canadian Council on Animal Care. Female Sprague-Dawley rats were obtained from Charles River, Canada (Montreal, Quebec) and were housed in the facilities of the University of Alberta until experimentation at 12-15 months of age. Animals were ovariectomized as described in the previous chapters.

4.2.2 Experimental design

To investigate the effects of estrogen, ovariectomized rats were treated with placebo pellet (n=8) or an estrogen pellet (1.5 mg/pellet, 60 day release, Innovative Research of America; n=8), which results in maximal serum estrogen levels (~80 pg/mL) similar to that of intact cycling rats. Cycling rats (~4 months old; in pro-estrus; n=6) were used as a reference group. Moreover, to evaluate the role of TNFα, estrogen deficient rats were treated with either Etanercept (Etan, n=8), or placebo (subcutaneous injection of dd H_2O; Placebo, n=8) for 4 weeks prior to experimentation as previously described. Since results from placebo animals (injection or pellet) were similar, they were combined for the final analysis.

4.2.3 Vascular function studies

Vessel preparation was done as described in chapter 2. Sensitivity of mesenteric arteries to vasoconstriction was evaluated with phenylephrine (PE). Cumulative concentrations of PE (0.1 to 50 μmol/L) were added to the bath and force was measured. After completion of each dose-response curve, a 30-minute recovery period was allowed,

during which the baths were changed every 10 minutes with fresh HEPES-PSS. To investigate the modulation of PE constriction by ANG II, PE constriction curves were generated in the absence or presence of the Candesartan (10 µmol/L; Astra Zeneca, Mississauga, Ontario), a specific AT_1R blocker, for 15 minutes prior to PE concentration-response curves.

The concentration of AT_1R blocker was calculated based on previous studies (17, 18). Moreover, in a subset of vessels pre-incubated with different concentrations of Candesartan (0.001 to 10 µmol/L), maximal effect on PE vasoconstriction was seen at 1 and 10 µmol/L. Moreover, since ANG II induces only a transient constriction in mesenteric arteries,(19) it was not possible to construct concentration-response curves of the direct vasoconstrictor effect of ANG II. The role of endothelium on PE responses and on ANG II modulation of constriction was evaluated by mechanically removal of the endothelium threading a human hair through the lumen of the artery. Confirmation of complete endothelium removal was assessed pharmacologically with a bolus dose of 1 µmol/L methacholine. All constriction curves were normalized to 100% for individual vessels

4.2.4 Western Blot analysis for AT_1R and ACE

Western blot for AT_1R and ACE protein expression in mesenteric arteries was performed as described in chapter 2. Twenty micrograms of protein were loaded onto a SDS-PAGE 9 % gel and transferred to a nitrocellulose membrane. Membranes were then probed with rabbit polyclonal anti AT_1R antibody (1:400; Santa Cruz Biotechnology) or anti ACE antibody (1:200; Santa Cruz Biotechnology). Specificity of primary antibodies

was tested using a specific blocking peptide (*Santa* Cruz Biotechnology). Primary antibody was preabsorbed for 30 minutes with a five times higher concentration of specific blocking peptide before probing the membranes. The primary antibody was then detected with a peroxidase-conjugated host specific secondary antibody (1:2000; Santa Cruz Biotechnology). Membranes were scanned with a Fluor Multimager and bands were quantified by densitometric analysis. After initial exposure to these antibodies, membranes were washed three times with 0.1 % TPBS, and then probed with anti α-tubulin (as a loading control; 1:1000; Santa Cruz Biotechnology).

4.2.5 Immunofluorescence for ANG II expression on mesenteric arteries.

Mesenteric arteries were placed in embedding medium (OCT; Tissue-Tek) and frozen in liquid nitrogen. Arteries were sectioned (10 μm) using a cryostat and fixed with cold acetone. Slides were then incubated with and without a primary antibody against ANG II (1:100; Peninsula Laboratories, CA) and then exposed to a fluorescent secondary antibody (1:200; Alexafluor 488; Molecular Probes, Leiden, The Netherlands). The Vectashield H-1200 Mounting Kit (Vector Laboratories, Burlingame, CA) was used and slides were analyzed under a fluorescence microscope (Olympus).

4.2.6 Data Analysis

Data from each dose-response curve was fitted to the Hill equation, and a straight line generated by linear least-squares regression analysis. The concentration that would give 50% constriction (EC_{50}) for each individual artery was determined from this line and

the mean ± SE calculated from the curves. Tension (T) was calculated using the formula: T=Force (milliNewtons [mN])/2 X axial length (mm^2). Analysis of variance (ANOVA) was used for statistical analysis among groups. Post hoc analysis was performed using Tukey's test. A Student's *t* test was used to compare EC_{50} between two groups. Tests were considered significant at $P<0.05$.

4.3 RESULTS

4.3.1 Effects of estrogen and TNFα inhibition on PE vasoconstriction

TNFα inhibition with Etanercept decreased the sensitivity to PE vasoconstriction in estrogen deficient animals (Placebo vs. Etanercept: EC_{50}= 2.2 ± 0.1 vs. 4.7 ± 0.7 μmol/L; $P < 0.05$) to levels comparable to estrogen-replaced (EC_{50}= 3.9 ± 0.4 μmol/L) and similar to that of cycling rats (EC_{50}= 4.7 ± 0.7 μmol/L). However, there were no differences in maximum tension among groups (Placebo, Estrogen and Etanercept: 3.9 ± 0.7, 3.8 ± 0.5 and 3.9 ± 0.7 mN/mm^2, respectively)

4.3.2 Effects of AT_1R blockade on PE vasoconstriction

In vitro AT_1R blockade with Candesartan (10 μmol/L) decreased PE sensitivity of vessels from estrogen deficient rats (EC_{50} = 4.4 ± 0.6 μmol/L; Figure 4.1), to levels similar to that of estrogen-replaced (EC_{50}= 3.9 ± 0.4 μmol/L) or etanercept (EC_{50}= 4.7 ± 0.7 μmol/L) treated animals. Similar results were obtained in placebo vessels preincubated with an Angiotensin Converting Enzyme (ACE) inhibitor (Captopril; 1 μmol/L) for 30 minutes (EC_{50} = 3.64 ± 0.9 μmol/L). However, Candesartan did not

significantly alter PE constriction in cycling animals (EC_{50} = 4.1 ± 0.2 μmol/L) or in estrogen depleted animals treated either with estrogen replacement (EC_{50} = 2.8 ± 0.4 μmol/L; Figure 4.1B) or Etanercept (EC_{50} = 4.27 ± 0.3 μmol/L; Figure 4.1C). Candesartan did not alter maximum tension in placebo, estrogen or Etanercept treated animals (3.5 ± 0.8, 4.0 ± 0.7 and 4.21 ± 0.9 mN/mm2 respectively).

4.3.3 Effects of endothelial removal on Candesartan Modulation of PE vasoconstriction in estrogen deficient animals

Since the endothelium could be involved in the generation of ANG II (20) that we observed in placebo animals, we next tested the effects of endothelial denudation. Endothelial removal did not significantly modify the effects of Candesartan (EC_{50}; with vs. without endothelium = 4.40 ± 0.6 μmol/L vs. 4.51 ± 0.7 μmol/L; Figure 4.2), indicating that the presence of an intact endothelium is not necessary for the effect of AT_1R blockade on modulation of PE constriction.

4.3.4 Effects of estrogen and TNFα inhibition on AT_1R, ACE and ANG II expression in mesenteric arteries

The expression of AT_1R and ACE in mesenteric arteries was evaluated by Western Immunoblot. Vascular expression of AT_1R and ACE was higher in estrogen deficient rats compared with either estrogen replaced or TNFα inhibition (Figure 4). ANG II expression in mesenteric arteries was evaluated by immunofluorescence. ANG II fluorescent staining was primarily located in the arterial media, and was higher in

estrogen-depleted animals compared with treated estrogen and etanercept treated groups (Figure 4.4).

4.4 DISCUSSION

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine involved in the pathogenesis of cardiovascular disease (21, 22). Interactions between TNFα and the RAS has been shown to occur in the cardiovascular system (2, 16, 23). In this study, selective TNFα inhibition reduced vascular expression of ACE, ANG II and AT_1R in mesenteric arteries of estrogen deficient rats. Importantly, these changes were accompanied by a decrease in the modulation of vasoconstriction by AT_1R blockade suggesting that TNFα may be a mediator of the alterations of vascular RAS during estrogen deficiency.

Previous studies have reported that estrogen deficiency is associated with an increase in vasoconstriction (2, 16, 24, 25), which is in part mediated by an increase in the production of endogenous vasoconstrictors (2, 16). In this study we sought to investigate the contribution of endogenous ANG II to vasoconstriction in the estrogen deficient rat using the adrenergic agonist phenylephrine. ANG II is a vasoconstrictor that can be generated in vascular tissues. All components for ANG II formation (renin, angiotensinogen, and ACE) can be found in the vascular wall (26), and ANG II formation has been demonstrated to occur in endothelial cells (27) as well as in VSMC (28, 29).

Endogenous ANG II mediates adrenergic constriction in different vascular beds (6, 7, 30-32) including mesenteric arteries (6, 7, 33). Accordingly, vasoconstriction to adrenergic agonists such as phenylephrine is modulated by ANG II receptor blockers (6, 7, 30, 34) or ACE inhibitors (17, 31, 33). However, whether endogenous ANG II modulates vascular tone during the state of estrogen deficiency was unknown.

We first investigated the effect of AT_1R blockade on PE vasoconstriction and found that pre-incubation of isolated mesenteric arteries with a selective AT_1R blocker (Candesartan) decreased the sensitivity to PE constriction in vessels from estrogen-depleted animals, but did not have any significant effect in vessels from cycling animals, estrogen-replaced animals or estrogen deficient animals treated with a TNFα inhibitor. Similar results were obtained in a subset of vessels from placebo animals pre-incubated with an ACE inhibitor (Captopril). These results may suggest that endogenous ANG II contributes to enhance vasoconstriction in vessels from estrogen deficient animals, and that this effect was attenuated by estrogen replacement or chronic TNFα inhibition.

Next, we investigated ANG II expression in mesenteric arteries. Interestingly, ANG II staining was increased in vessels from estrogen deficient animals compared with estrogen replaced-animals or estrogen deficient animals treated with a TNFα inhibitor. ACE is involved in the local conversion of ANG I into ANG II, and studies have shown that the level of ACE expression in vascular tissues, correlates with the rate of local ANG II formation (4, 35). In the present study, estrogen deficient animals have a ~3 fold-increase in ACE expression compared with either estrogen replaced animals or Etanercept treated animals. These results agree with previous studies reporting that estrogen modulates ACE expression (8), and importantly, they suggest that TNFα participates in the regulation of ACE vascular expression during estrogen deficiency.

Since endothelial cells are a source of potent vasoconstrictors such as ANG II, we tested whether the endothelium could be the primary source of endogenous ANG II. However, endothelial removal did not alter the effects of AT_1R blockade on constriction suggesting that the endothelium is not necessary for ANG II formation. Accordingly,

ANG II staining in vessels from estrogen deficient animals was positive across all vascular layers. In agreement with these observations, Leite et al found that endothelium-denuded mesenteric arteries were still able to produce ANG II (36).

AT_1R mediates some of the detrimental effects on vascular function attributed to ANG II such as oxidative stress as well as vasoconstriction (37). Moreover, previous studies have suggested that AT_1R expression is altered by TNFα or estrogen. Recently, Hinojosa-Laborde et al (38) reported that in salt sensitive rats, ovariectomy increases AT_1R binding in the kidney and that it was associated with an increase in blood pressure, which were reduced by estrogen replacement. However, the role of estrogen and TNFα on AT_1R expression on resistance arteries and its association with changes in vascular function was unclear. We observed that estrogen deficient animals had a ~ two-fold increase in AT_1R expression compared with estrogen-replaced animals or Etanercept treated animals. These observations suggest that the upregulation of vascular AT_1R during estrogen deficiency may contribute to enhance vasoconstriction. Indeed, the reduction in AT_1R by estrogen or TNFα antagonism was also associated with a decrease in both PE sensitivity and Candesartan modulation of PE constriction. Thus, the increase in ANG II modulation of vasoconstriction in estrogen deficient rats could be mediated by both an increase in its vascular formation and greater expression of AT_1R.

These results also indicate that an increase in TNFα levels during estrogen deficiency may result in higher vascular AT_1R expression. In fact, in isolated rat VSMC (data not shown) as well as in other cell types (39) TNFα was able to up-regulate AT_1R expression. In summary, estrogen deficiency was associated with higher vascular expression of ACE, ANG II and AT_1R as well as an increase in PE vasoconstriction,

which was attenuated by *in vitro* AT_1R blockade or ACE inhibition. Moreover, estrogen replacement or TNFα inhibition reduced the expression of ACE, ANG II and AT_1R and these changes were accompanied by a decrease in the sensitivity to vasoconstriction and a lack of modulation by AT_1R blockade. Taken together these observations suggest that estrogen deficiency is associated with an increase in ANG II modulation of vasoconstriction and that TNFα may mediate vascular dysfunction during estrogen deficiency by promoting the effects and/or the formation of ANG II.

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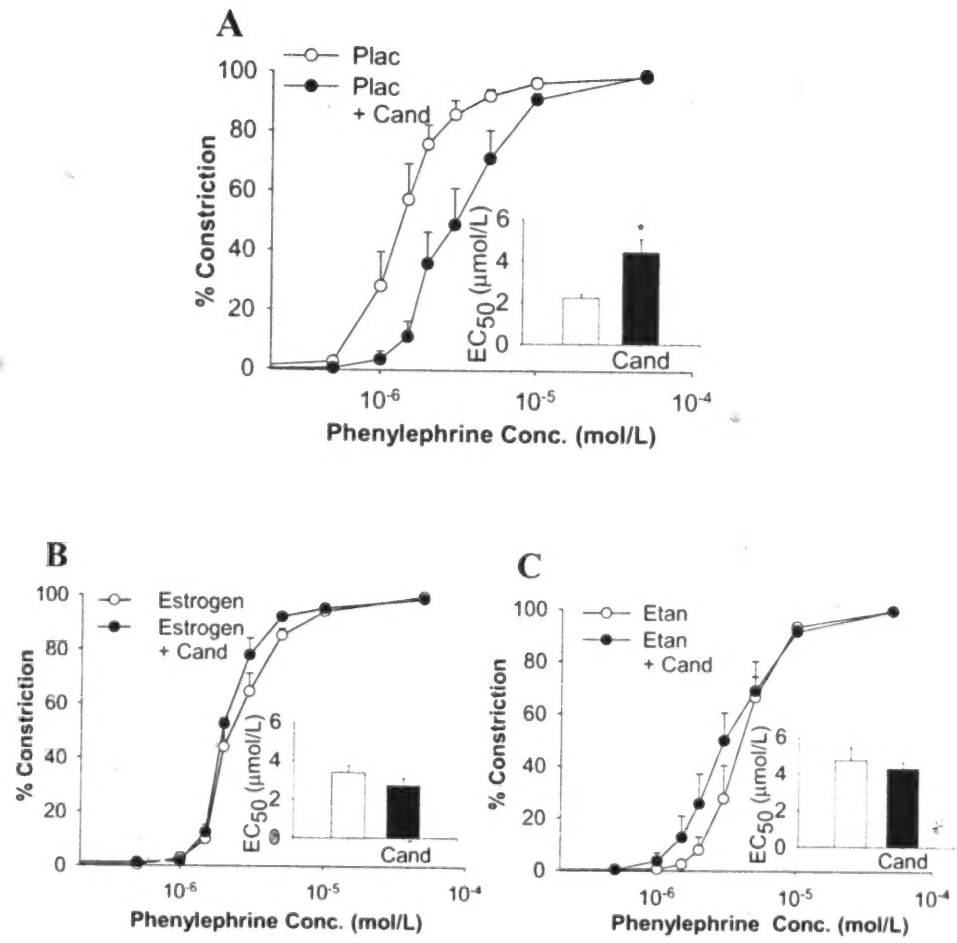

Figure 4.1 **AT_1R blockade with Candesartan decreased PE sensitivity of vessels from estrogen deficient rats to levels similar to that of estrogen-replaced or etanercept treated animals.**

Effects of AT_1R blockade with Candesartan on phenylephrine (PE) constrictor responses of mesenteric arteries. PE concentration response curves and EC_{50} bar graphs in the absence (o) or presence (•) of Candesartan (Cand; 10 µmol/L) of mesenteric arteries from ovariectomized rats treated either with placebo (Plac, n=8; Panel 2A), estrogen (n=8; Panel 2B) or Etanercept (Etan; n=8; Panel 2C). Bars represent mean ± SE. * P <0.05 vs. PE alone.

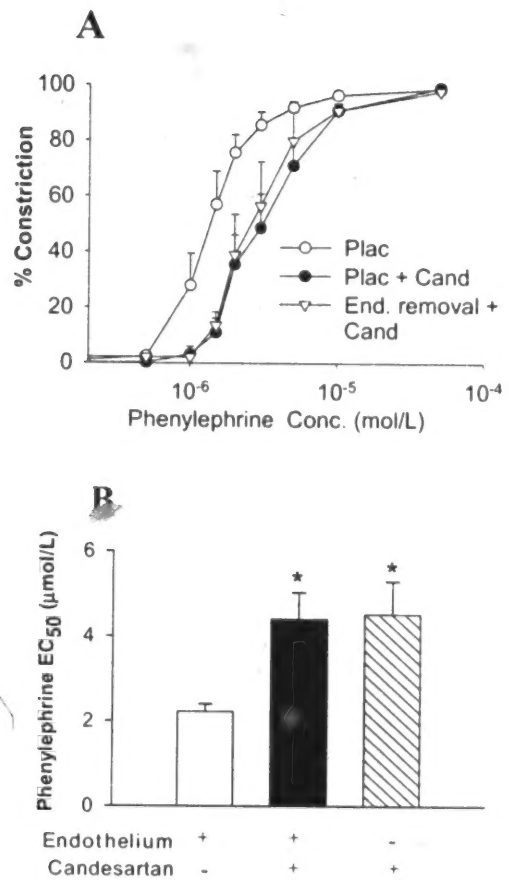

Figure 4.2 The decrease in vasoconstriction by Candesartan is not mediated by endothelial factors.

Effects of endothelial removal on AT_1R modulation of phenylephrine vasoconstriction in estrogen deficient animals. (A) PE concentration response curves and (B) EC_{50} bar graphs of mesenteric arteries from ovariectomized rats treated with placebo in the presence of endothelium (Plac; n=15), with endothelium plus Candesartan (Plac + Cand; n=8) and without endothelium plus Candesartan (n=4). Bars represent mean + SE. * $P<0.05$ vs. intact vessels.

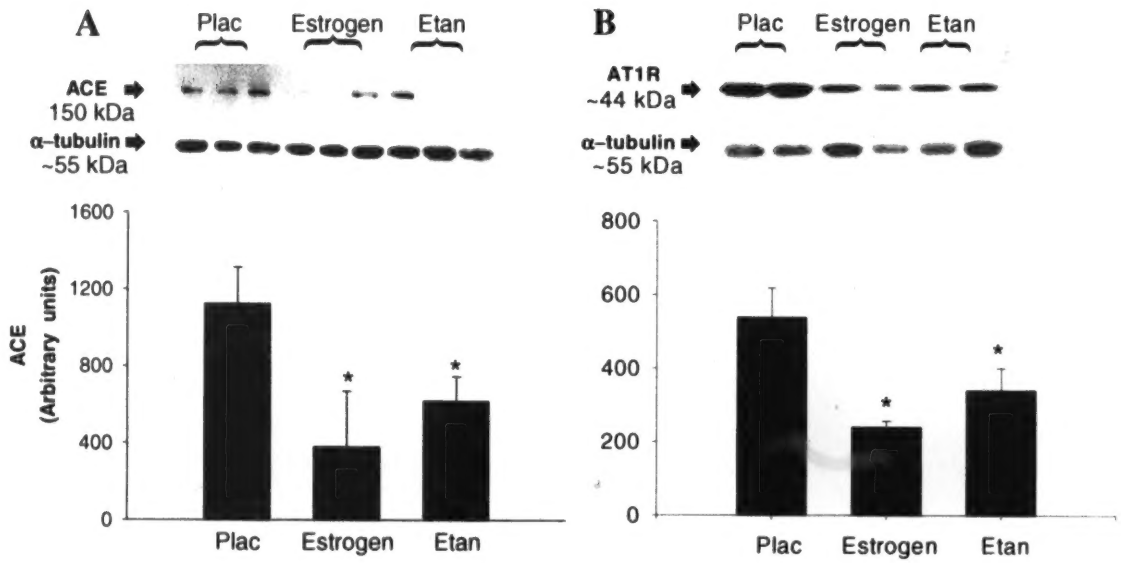

Figure 4.3 Estrogen replacement or TNFα inhibition decreases the vascular expression of ACE and AT_1R.

Angiotensin Converting Enzyme (ACE) and Angiotensin type I receptor (AT_1R) expression in mesenteric arteries. Representative Western blots and densitometric analysis for ACE (A) and AT_1R (B) expression in arteries from placebo (Plac; n=5), estrogen (Estrogen; n=5) or Etanercept treated animals (Etan; n=5). Bars represent mean + SE. * $P<0.05$ vs Placebo

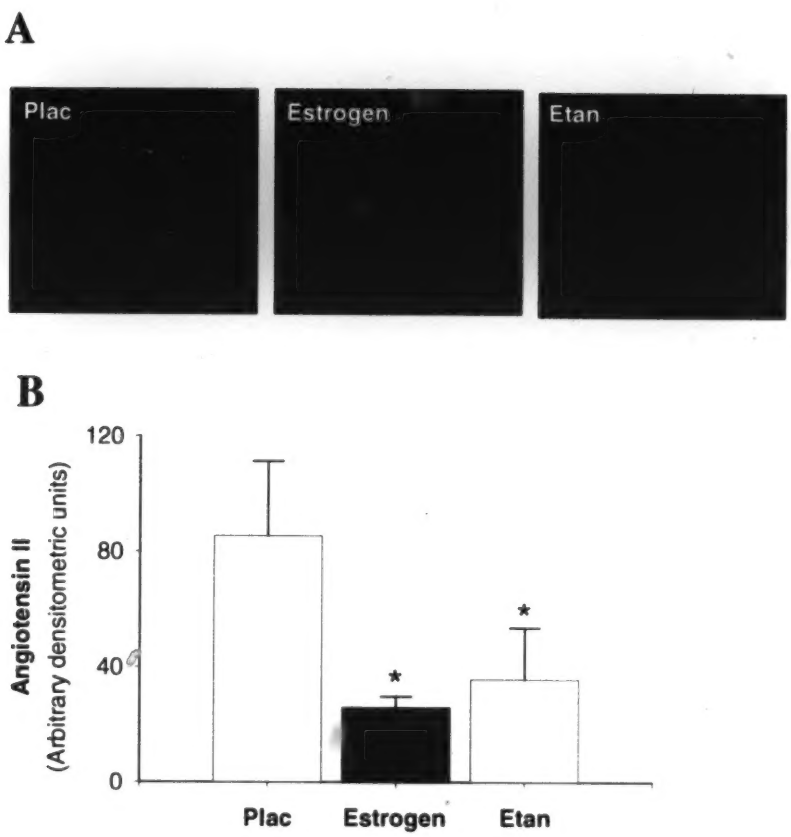

Figure 4.4 Treatment with Etanercept or estrogen reduces ANG II vascular expression in aged rats.

Immunofluorescence for ANG II expression in mesenteric arteries from estrogen deficient rats (Plac) treated either with estrogen replacement (Estrogen) or chronic TNFα inhibition (Etanercept; Etan). Pictures are representative of 4 independent experiments. Vessels were probed with fluorescent antibodies against Angiotensin II (Red). Blue color is DAPI staining for cell nuclei. Green color is auto-fluorescence and represents the arterial elastic internal lamina and vessel wall. B. Densitometric analysis of fluorescence intensity. Bars represent means ± SE. * $P<0.05$ vs Placebo.

CHAPTER 5. ANGIOTENSIN II-INDUCED MMP-2 RELEASE FROM ENDOTHELIAL CELLS IS MEDIATED BY TNFα

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5.1 INTRODUCTION

Angiotensin II (ANG II) is an important modulator of vascular homeostasis, and an important link in the pathophysiology of cardiovascular disease (1, 2). Elevated ANG II and/or increased sensitivity to ANG II have been etiologically associated with major vascular diseases (1, 2). However, most of the studies concerning the effects of ANG II on vascular cells have been conducted in smooth muscle cells. Recently, an emerging role for ANG II is through modulation of endothelial cell function. Endothelial cells are essential to maintain normal vascular tone, blood fluidity and to limit vascular inflammation (3). Indeed, a common feature of vascular disorders is the presence of endothelial dysfunction.

Tumor necrosis factor alpha (TNFα) has been proposed to be an important mediator of the endothelial alterations seen in vascular disease (4, 5). TNFα stimulated endothelial cells undergo functional alterations resulting in a pro-thrombotic and pro-inflammatory phenotype (activation) (6). TNFα levels are elevated in a number of vascular disorders, and it appears to be involved in the chronic development of atherosclerosis, as well as in the acute plaque events that can result in clinical events such as myocardial infarction or

stroke (7, 8). TNFα is principally derived from mononuclear phagocytes, but it can be also synthesized in vascular cells such as smooth muscle and endothelium (6). The role of TNFα produced in vascular cells is not very well understood; however, it is likely to modulate key vascular processes such as angiogenesis and inflammation (4-6). Importantly, endothelial-derived TNFα could contribute to the pathogenesis of vascular disease.

Interactions between ANG II and TNFα may play an important role in the modulation of endothelial function. Some studies have suggested that TNFα could mediate the vascular effects of ANG II (9). Interestingly, in endothelial cells, some of the effects of TNFα, such as increased free radical production, inflammation and enhanced remodeling, resembled those attributed to ANG II. Moreover, ANG II has been reported to activate pro-inflammatory transcription factors in endothelial cells, known to induce the formation of TNFα (10). Furthermore, ANG II has been shown to stimulate the production of TNFα on other vascular cells (11). Altogether, these observations suggest that TNFα could mediate some of the effects of ANG II on endothelial function.

Proinflammatory cytokines such as TNFα have been shown to induce the release of Matrix metalloproteinases (MMPs), including MMP-2 (7, 12). MMPs are a group of zinc-dependent endopeptidases that play a key role in matrix turnover. Indeed, increased interstitial matrix remodeling is believed to be involved in the pathogenesis of atherosclerosis and other vascular disorders (13, 14). MMP-2 participates in the breakdown of collagen type IV, a major component of subendothelial basement membrane (15, 16). Moreover, we have previously reported that MMP-2 through

cleavage of endothelium-derived peptides may also lead to vasoconstriction and inflammation (17, 18).

Enhanced MMP-2 activity has been shown to occur in vulnerable atherosclerotic plaques (19). Moreover, higher MMP-2 levels have been reported in patients after acute atherosclerotic events, and in women with preeclampsia (20). All these conditions have been associated with both increased effects of ANG II and higher levels of TNFα. However, whether ANG II could induce the release of MMP-2 from endothelial cells is unclear. In this study we evaluated the effects of ANG II on TNFα and MMP-2 release from endothelial cells. We hypothesized that ANG II could induce the release of MMP-2 from endothelial cells, in part through the formation of TNFα.

5.2 METHODS

5.2.1 Reagents

Angiotensin II, PD123319, and recombinant TNFα were purchased from SIGMA, while Candesartan was obtained from Astra Pharma Inc. Pentoxifylline (#002323-Hoechst Canada) was donated by A. Rabinovitch's laboratory, Edmonton, Canada. Neutralizing antibodies anti-human TNFα were obtained from ICN Biomedials, Inc. M199 medium , L-glutamine and trypsin were purchased from Life Technologies. Fetal bovine serum (FBS) was purchased from GIBCO, and endothelial growth factor (ECGF) from VWR international.

5.2.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords as previously described (21). Briefly, the cords were cleaned with PBS, and then incubated with collagenase A for 15 minutes at 37 °C. Endothelial cells were collected by centrifugation, and the pellet was resuspended in M199 with 20% FBS. Cells were grown on 0.1% gelatin coated-dishes in M199 medium with ECGF, heparin, and 20% FBS, and used at passages 2 to 4. Prior to stimulation HUVECs were plated in 6 well plates and incubated in serum free-M199 (without phenol red) containing 0.1% BSA and without ECGF. After stimulation with the different agonists, conditioned media were collected, cellular protein extracts were prepared or total cellular RNA was extracted.

5.2.3 Measurement of MMP-2 release

MMP-2 release from HUVECs was evaluated in conditioned media by gelatin zymography as previously described (12). Briefly, supernatants were collected and protein content was determined by the Bradford method. 0.5 µg of protein was loaded per lane and subjected to 7.5% SDS-PAGE copolymerized with gelatin (2.5mg/ml). After separation the gels were incubated with Triton X-100 (0.1%; 3 times, 20 min) at room temperature. Subsequently, gels were incubated in enzyme assay buffer containing (mM) 25 Tris, 5 $CaCl_2$, 142 NaCl, and 0.5 Na_3N at 37°C for 48 hours. To reveal zones of degradation gels were stained with comassie blue overnight and then in distain solution for 6 hours. Pure human MMP-2/MMP-9 zymography standards (Chemicon) were used as a control. Gels were scanned using Fluor Multimager (Bio-Rad). Preliminary experiments showed that MMP-2 activity detected in conditioned medium by

zymography correlated with MMP-2 prótein content detected by Western immunoblot (data not shown).

5.2.4 Determination of TIMP-2 release

To measure the release of TIMP-2, the tissue inhibitor of MMP-2, a commercial immunoassay (R & D systems) was carried out according to the manufacture's instructions. This solid phase ELISA enables measurements of total human TIMP-2. Supernatants were tested by duplicated for each sample. Absorbance at 450 was measured by an automated reader (Hewlett Packard).

5.2.5 Measurement of TNFα

In order to distinguish bioactive TNFα from that bound to Etanercept (inactive), serum TNFα was measured using the L929-8 bioassay, which allows distinguishing between free (bioactive) or bound forms of TNFα (22). Briefly, L929-8 cells (an isolated subclone of the murine fibroblastoid cell line L929) were cultured in the wells of a 96-well flat-bottomed microtiter plate in medium (IMDM) containing 10% FBS plus 2 µg/mL Actinomycin D (Kindly donated from the laboratory of Dr. Larry Guilbert, University of Alberta) for 2 hours at 37° in 5% CO_2 in air. Fifty microliters of serum or recombinant TNFα standards (1.56 to 200 pg/mL) were then added in triplicate to appropriated wells and incubated at 40°C for 20 hours. Cell viability was assessed by incubation for 2 hours with neutral red dye (0.05% in PBS), which is taken up by vital cells. Then, supernatant vial is removed, the adherent cells are washed with PBS, and color is developed with 0.05 M NaH2P04 in 50% ethanol. The optical density at 570nm

of each well, which reflects the number of cells still viable, was measured on an automated microplate reader. The concentration of TNFα in the sample can then be calculated by comparison with a standard curve constructed with the TNFα standards. The lower limit of assay sensitivity is approximately 200 fg/mL of pure, recombinant TNFα. This bioassay also detects TNFβ, but it is not affected by other known cytokines.

5.2.6 Western Blot analysis for TNFα

TNFα protein expression in endothelial cells was determined by Western blot. Briefly, cell lysates from stimulated HUVECs were collected with lyses buffer: 25 mM Tris-HCl pH 7.5 with 0.5% Triton X-100, and sonicated for ~5 sec. Protein content was determined by Bradford protein assay. Eighty micrograms of protein were loaded into a SDS-PAGE 15% gel and transferred to a PVDF membrane. Membranes were then probed with goat polyclonal anti human TNFα antibody (1:500) (Santa Cruz Biotechnology). The primary antibody was then detected using a peroxidase conjugated donkey anti-goat secondary antibody (1:2000) (Santa Cruz Biotechnology). Membranes were scanned using Fluor Multimager (Bio-Rad).

5.2.7 Measurement of TNFα mRNA

Levels of TNFα mRNA were analyzed by real time RT-PCR. The mixture of primers and probe specific for human TNF alpha were purchased from Applied Biosystems. Cell lysates from 3 different experiments were analyzed. 1μg of total RNA was reverse transcribed (RT). The RT protocol was performed according to the manufacturer's instructions (Byosistems, CA). The RT reaction consisted of 1 μg total

RNA, 1 X RT buffer, RNAase free water, 2.5μM of random hexamers, 0.4 U/ μL of RNAase inhibitor, 5.5mM of $MgCl_2$, 10 mM of dNTP mix, and 1.25 U/μL of MultiScribe™ Reverse Transcriptase. After incubation at 25°C for 10 min, and 60 min at 48°C, the RT enzyme was inactivated by heating at 95°C for 5 min.

The PCR mix consisted of 4 or 2 μL cDNA from RT reaction, Taqman® universal PCR master mix containing: AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs with dUTP (Applied Biosystems, catalog number 4304437), the mixture of primers and TaqMan® probe for human TNFα, and RNAase free water. Experiments were performed in triplicate for each sample. The PCR mix was initially heated to 50°C for 2 min and then at 95°C for 10 min for optimal Amperase UNG enzyme activity, and to activate AmpliTaq Gold DNA polymerase, followed by 40 cycles of 90°C for 30 s, 60°C for 60 s, and 72°C for 30 s. Average of cycle threshold (ΔCt) values for ANG II (0.1, 1, 10 μM) were compared with control.

5.2.8 Statistical Analysis

Results are expressed as a percentage of control. HUVECs obtained from different umbilical cords were considered as individual experiments. ANOVA one way or in ranks were used to evaluate differences among groups, and Tukey test for post hoc analysis. Values of $P < 0.05$ were considered significant.

5.3 RESULTS

5.3.1 Effects of Angiotensin II on MMP-2 and TIMP-2 release

To evaluate the effects of ANG II on MMP-2 and TIMP-2 release, HUVECs were stimulated with ANG II (0.1 to 10 μM) for 24 hours. ANG II stimulation resulted in increased MMP-2 release (Figures 5.1A and 5.1B). Moreover, to determine the role of AT_1R and AT_2R, cells were pre-treated with antagonists of AT_1R (Candesartan; 100 μM) or AT_2R (PD123319; 100 μM) one hour before ANG II stimulation. Pre-treatment with Candesartan but not with PD123319, prevented the secretion of MMP-2 induced by ANG II (Figures 5.2A and 5.2B). In similar cell culture conditions, ANG II did not affect the secretion of MMP-9 (92 kDa). Furthermore, ANG II reduced the secretion of TIMP-2 from endothelial cells, which was also inhibited by AT_1R antagonism (Figure 5.3). AT_2R antagonism did not affect ANG II-induced TIMP-2 release.

5.3.2 Effects of Effects of ANG II on TNFα formation

TNFα is first synthesized as an immature peptide (pro-TNFα) that is later cleaved by a TNFα converting enzyme (6). After treatment with ANG II for 24 hours (0.1 to 10 μM), TNFα protein was evaluated in cell lysates with an antibody able to detect mature and immature forms of TNFα. We found pro-TNFα and mature TNFα protein levels to be significantly higher in cells stimulated with ANG II compared to control (Figures 5.4A and 5.4B). The effect of ANG II on TNFα mRNA transcription was evaluated by real time RT-PCR. RNA isolated from control and ANG II-treated cells was reverse transcribed, and first-strand cDNA was further amplified. The average threshold for ANG II-treated cells occurred earlier than in controls (ANG II 0.1μM: 32.2 ± 0.1; 1μM: 30.7 ± 0.3; 10μM: 30.01 ± 0.1 vs. control: 35.6 ± 0.4) ($P<0.05$) (Figure 5.5), indicating that ANG II induces the formation of TNFα mRNA in HUVECs.

To elucidate if the increments of TNFα mRNA and protein were translated into release of bioactive TNFα, cell culture supernatants from cells treated with and without ANG II were tested using the L929-8 bioassay. We found that ANG II (10 μM) increased the amount of bioactive TNFα in supernatants, which was prevented by pre-treatment with Candesartan (Figure 5.6).

5.3.3 Effects of TNFα inhibition on ANG II induced MMP-2 release

To evaluate whether TNFα could mediate the effect of ANG II on MMP-2 release, cells were first pretreated with neutralizing antibodies against human TNFα one hour before ANG II stimulation. The concentration of anti-TNFα antiserum was calculated based on the ability to neutralize the effects of 50 pgm of TNFα on L929 cells. TNFα blockade significantly reduced ($P<0.05$) the secretion of MMP-2 induced by ANG II (Figures 5.7A and 5.7B). The use of antiserum alone did not affect the basal release of MMP-2. Moreover, pre-treatment with pentoxifylline (0.1 and 1 mg/mL), a non-selective phosphodiesterase inhibitor that has been shown to inhibit TNFα synthesis also prevented the release of MMP-2 induced by ANG II (reduction of 40 ± 10 and 110 ± 9 % respectively, compared to ANG II alone, $P< 0.05$). Pentoxifylline (1 mg/mL) did not significantly change the basal release of MMP-2 from endothelial cells.

5.3.4 Discussion

Our data indicate that ANG II-induced release of endothelial MMP-2 is mediated by TNFα. It has been shown that ANG II may induce a pro-inflammatory phenotype in

endothelial cells (i.e. increase the expression of adhesion molecules such as ICAM-1) (23). However, to our knowledge, there are no previous studies reporting the effects of ANG II on TNFα formation in endothelial cells. The present study shows evidence of TNFα generation at the level of mRNA, protein expression and function. These observations are clinically relevant, due to the key role of ANG II and TNFα in the pathogenesis of vascular disorders such as preeclampsia and atherosclerosis, and the availability of treatments to antagonize these factors.

In fact, recent studies have shown TNFα antagonism improves endothelial function in patients with chronic heart failure (24) or chronic inflammation (25). Although the concentration of ANG II that results in a maximum effect on TNFα production is higher than the reported circulating levels, it is likely that circulating levels underestimate the concentration available in vascular beds. For instance, there is evidence that locally formed (within vascular walls) ANG II may account for part of its vascular effects (26). The fact that ANG II may induce the formation of active TNFα in endothelial cells is intriguing. ANG II induced TNFα gene expression as well as the formation of immature and bioactive forms of TNFα, which suggest that ANG II stimulates the proteolytic cleavage of pro-TNFα. TNFα is an inflammatory cytokine proposed to be a mediator of the endothelial alterations seen in cardiovascular disease (5, 6). Indeed, TNFα has been shown to induce endothelial dysfunction, inflammation and apoptosis (4-6). Interestingly, some of the effects of TNFα on endothelial function, such as increased free radical production, inflammation and enhanced remodeling, resembled those attributed to ANG II. On the other hand, TNFα has been shown to protect endothelial cells from apoptosis

by inducing platelet-derived growth factor pathways (27). Therefore, endogenous production of TNFα could trigger either detrimental or protective pathways to modulate the effects of ANG II on endothelial cells. Understanding the role of interactions between ANG II and TNFα on endothelial modulation of vascular function is necessary.

MMP-2 is an important protease involved in normal (angiogenesis or wound repair) and pathologic blood vessel remodeling (chronic inflammation and tumor genesis)(15, 16). In this study, we found that ANG II increases the release of MMP-2 from endothelial cells, while decreasing the secretion of TIMP-2 the endogenous inhibitor of MMP-2, which was prevented by AT_1R antagonism. Therefore the increased MMP-2 activity after ANG II stimulation could be due both to increased MMP-2 release and decreased TIMP-2 secretion. These observations may indicate that ANG II via AT_1R could induce matrix turnover by enhancing the release-activity of MMP-2.

Interestingly, opposing findings have been reported in vascular smooth muscle cells and fibroblasts, where ANG II decreased the secretion of MMP-2 (28, 29). These observations suggest cell specific effect of ANG II, which is important to understand when using AT_1R antagonists clinically. It could be speculated that for smooth muscle cells and fibroblasts, ANG II mediated decrease in MMP-2 release could lead to collagen deposition promoting fibrosis, while in endothelial cells, ANG II induced-MMP-2 activity could be involved in other processes such as angiogenesis, thrombosis, and inflammation. Indeed, we have shown that MMP-2 through cleavage of endothelial-derived peptides can directly promote vasospasm and facilitate leukocyte recruitment (12, 17).

The present study suggests that a dysfunctional endothelium caused by ANG II, may directly affect the development and progression of atherosclerosis and other vascular disorders, by enhancing the local production of TNFα, a potent inflammatory cytokine postulated to be an important mediator of vascular disease. Although in most vascular disorders an upregulation of ANG II, TNFα, and MMP-2 have been described, the contribution of TNFα and MMP-2 to the pathophysiological effects of ANG II remains to be understood. Furthermore, these findings also suggest that MMP-2 and/or TNFα inhibition could have potential therapeutic implications in some vascular disorders where ANG II plays a role in the pathogenesis of vascular dysfunction.

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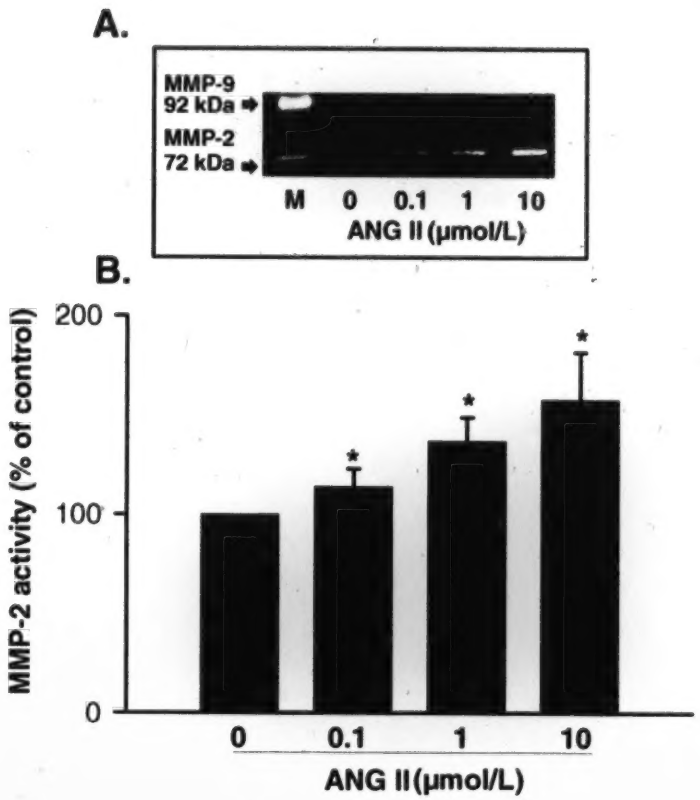

Figure 5.1 Angiotensin II induces endothelial release of MMP-2.

Panel A: Representative gel showing an increase in MMP-2 (72 kDa) activity in supernatants from HUVECs after ANG II stimulation. Panel B: Summary of densitometric analysis for MMP-2 activity (n=7). Data is presented as percentage of control (± SEM). *P<0.05 vs control.

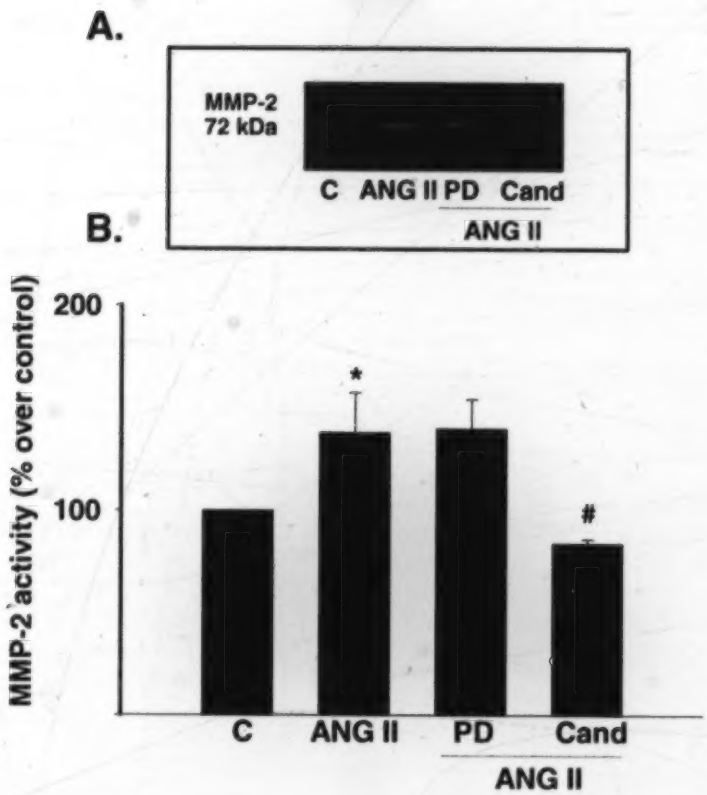

Figure 5.2 AT_1R but not AT_2R antagonism prevents MMP-2 release from HUVECs induced by Angiotensin II.

Panel A: Representative gel showing that pretreatment with the AT_1R antagonist Candesartan (Cand; 100 µmol/L), but not with PD123329 (PD; 100 µmol/L) an AT_2R antagonist, prevents the Angiotensin II (10 µmol/L) -induced MMP-2 release.
Panel B: Summary of densitometric analysis for MMP-2 activity (n=5). Data is presented as percentage of control (± SEM). *P<0.05 vs control; #P<0.05 vs ANG II alone.

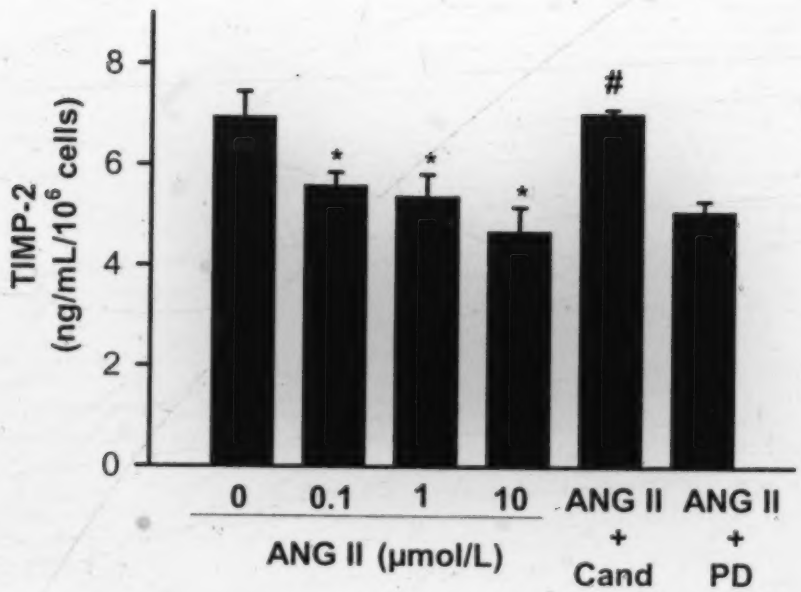

Figure 5.3 Angiotensin II reduces endothelial release of TIMP-2.

Human TIMP-2 was determined by immunoassay in HUVECs culture supernatants after 24 hour of stimulation with Angiotensin II (ANG II; 10µmol/L). The AT_1R antagonist Candesartan (Cand; 100 µmol/L) but not PD123329 (PD; 100 µmol/L), an AT_2R antagonist, prevented the release of MMP-2 induced by ANG II. Data showed are mean ± SE of 3 experiments. *P<0.05 vs control; #P<0.05 vs ANG II.

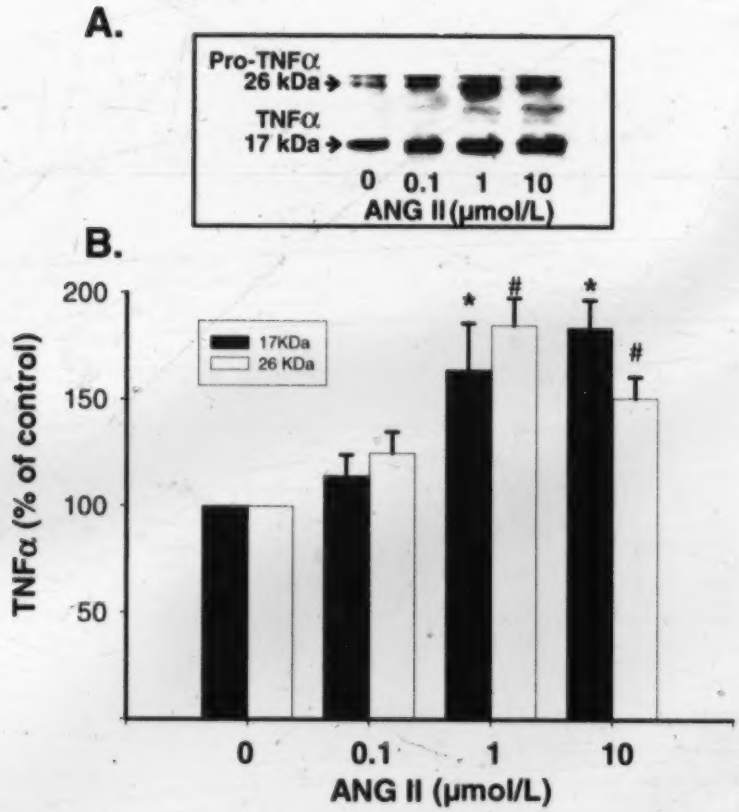

Figure 5.4 Angiotensin II enhances endothelial TNFα protein production.

Panel A: Representative Western immunoblot showing an increase in the expression of pro-TNFα (~26 kDa) and TNFα (~17 kDa) after ANG II stimulation.

Panel B: Summary of densitometric analysis for TNFα expression. Data is presented as percentage of control (± SEM) (n=3). *P<0.05 vs control (TNFα); #P<0.05 vs control (pro-TNFα)

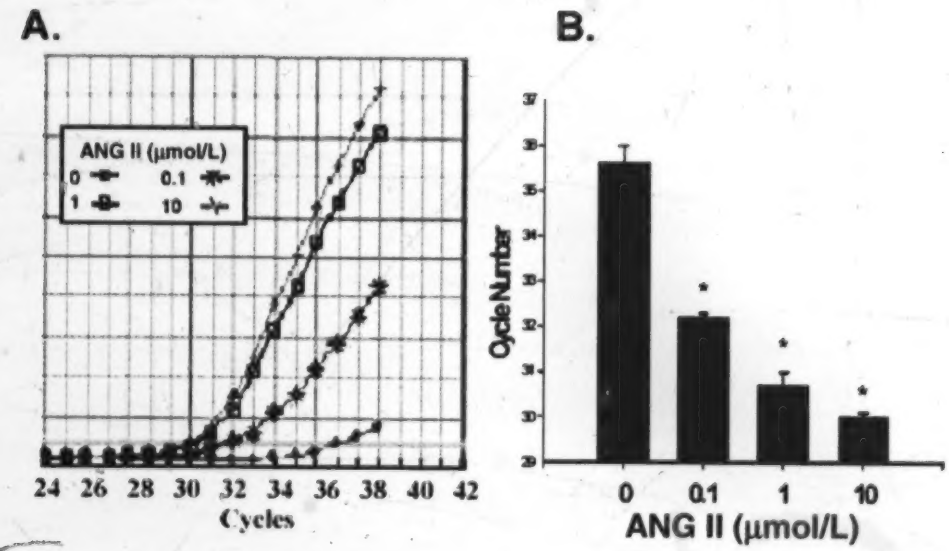

Figure 5.5 Angiotensin II enhances endothelial TNFα mRNA production.

Panel A. Representative real time RT-PCR, showing real-time signals for different concentrations of Angiotensin II (ANG II). The amplification was monitored by real-time PCR using green fluorescent dye. The fluorescent intensity is given in arbitrary units. ANG II samples were amplified for 40 cycles (Annealing temperature at 60°C). Cell lysates from 3 independent experiments were analyzed.

Panel B summarizes the number of cycles required to achieve threshold fluorescence (C_t). Lower C_t values in ANG II-treated cells (0.1µmol/L: 32.2 ± 0.1; 1µmol/L: 30.7 ± 0.3; 10µmol/L: 30.01 ± 1 vs. untreated: 35.6 ± 0.4) (*P<0.05 vs control) indicate an increase in TNFα mRNA expression compared to cells treated with medium alone. Experiments were done in triplicate using a negative control as a background

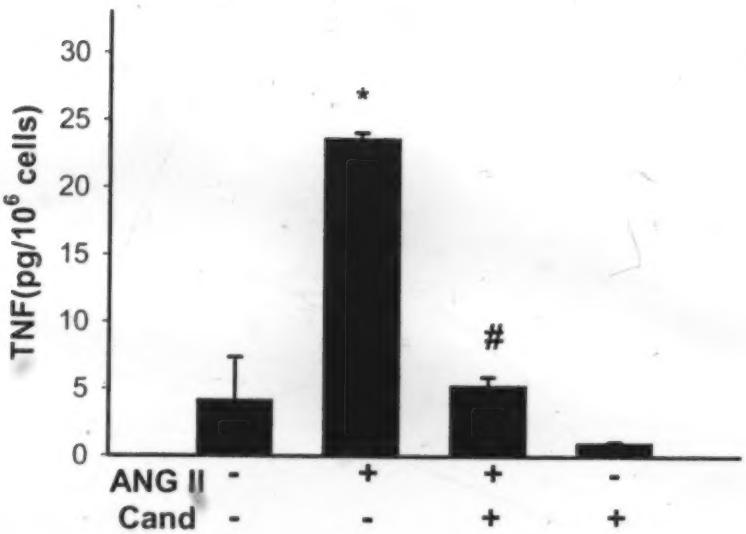

Figure 5.6 Angiotensin II enhances the release of bioactive TNFα from endothelial cells.

TNFα production (pg/10^6 cells) was quantified using the L929-8 bioassay by comparison to a pure, recombinant human TNFα. ANG II (10 μmol/L) increased the amount of bioactive TNFα in supernatants, which was prevented by pre-treatment with Candesartan (Cand; 100 μmol/L). Results represent the means of triplicate assays ± SD. The results of this experiment were similar to those obtained in two additional experiments. *$P<0.05$ vs control; #$P<0.05$ vs ANG II alone.

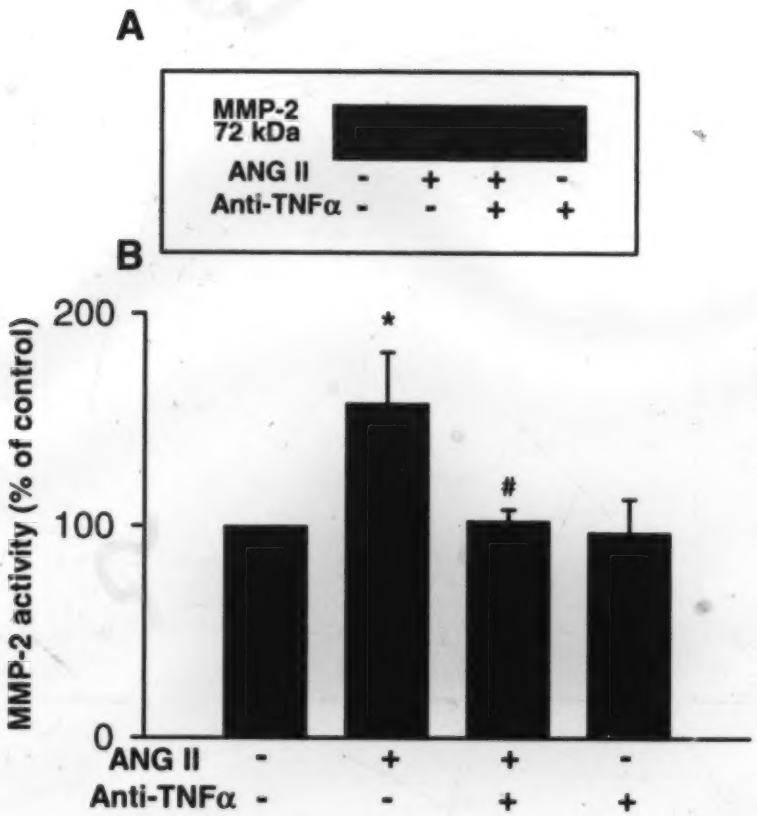

Figure 5.7 Antiserum against TNFα inhibits endothelial MMP-2 release induced by Angiotensin II.

Panel A: Representative gel showing that pretreatment with anti-TNFα antibodies (25 μg/mL) prevented the ANG II-induced MMP-2 release.

Panel B: Summary of densitometric analysis for MMP-2 activity (n=5). Data is presented as percentage of control (± SEM). *P<0.05 vs control. #P<0.05 vs ANG II (10μM).

CHAPTER 6. GENERAL DISCUSSION

6.1 SUMMARY

Inflammatory factors are associated with the pathogenesis of vascular dysfunction. Estrogen deficiency is associated with alterations on immune function. In this thesis I am presenting evidence that TNFα, a pro-inflammatory cytokine, is involved in the alterations in vascular function in a model of aging in female rats. Isolated resistance arteries were studied focusing on NO and ANG II pathways, which alterations have been associated with the pathogenesis of cardiovascular disease. The role of estrogen deficiency in aging was investigated by performing ovariectomy followed by estrogen replacement, and the role of TNFα was evaluated by using a selective TNFα inhibitor (Etanercept). Aged estrogen deficient rats have an increase in bioactive serum levels of TNFα compared with age-matched estrogen-replaced animals.

TNFα inhibition in estrogen deficient animals resulted in an increase in NO modulation of vasoconstriction, increased in agonist-induced endothelial-dependent vasodilation and flow-mediated vasodilation likely due to both an increae in NO synthesis and a decrease in NO inactivation by free radicals. Indeed, eNOS expression was increased by TNFα inhibition, whereas the expression of NAD(P)H oxidase was reduced. Furthermore, our findings also indicate that arteries from estrogen deficient animals have greater formation of ANG II, higher expression of AT_1R, and that ANG II is an endogenous vasoconstrictor that enhances adrenergic vasoconstriction in estrogen deficient animals. TNFα inhibition was associated with a decrease in vascular expression

of ACE, ANG II and AT_1R as well as with a reduction in ANG II-mediated vasoconstriction. These results suggest that interactions between TNFα and ANG II are involved in the alterations of vascular function during estrogen deficiency. The role of this interaction was further investigated in endothelial cells. ANG II was found to induce the formation and release of bioactive TNFα from endothelial cells via AT_1R. Furthermore, ANG II decreased TIMP-2 and induced the release of MMP-2 from endothelial cells, in part through the formation of TNFα. The primary findings of this thesis are summarized in Figure 6.1.

6.2 *VASCULAR ALTERATIONS IN A MODEL OF FEMALE AGING*

In order to understand the vascular changes that occur in postmenopausal women, we conducted experiments in female rats approaching reproductive senescence, which were ovariectomized to remove variable endogenous estrogen levels. These rats develop some of the vascular alterations described in postmenopausal women, and our laboratory has published extensively using this model (1-3, 15, 38, 44-46).

6.2.1 Effects of estrogen deficiency on TNFα levels

In our studies, aged estrogen deficient rats had an increase in bioactive serum levels of TNFα compared with estrogen-replaced animals. These results agree with some clinical studies reporting that TNFα levels are higher in postmenopausal women compared with premenopausal women (9, 23, 32, 37). Moreover, in clinical settings, several confounding factors such as age, co-morbidity and different genetic background,

may affect TNFα levels. Furthermore, in those studies vascular function was not evaluated and hence, whether TNFα could be a mediator of vascular dysfunction during estrogen deficiency was unknown. Thus, the results of our studies conducted under controlled conditions, indicate that estrogen deficiency results in a spontaneous increase in circulating TNFα levels. The experimental time was chosen based on our previous studies in this model, which suggest that 4 weeks of estrogen deficiency or replacement is an appropriate period of time to detect changes in vascular function (2, 3). Moreover, a spontaneous increase in TNFα release from monocytes is clearly evident after 2 weeks of oopherectomy and peaks around the fourth week (31). Furthermore, in animal models of inflammation (22) and in clinical trials for inflammatory conditions (29), the initial benefit of TNFα inhibition with Etanercept are seen after 2 weeks of treatment. We did not explore, however, whether longer periods of time after ovariectomy would normalize or further increase TNFα levels. In post-menopausal women TNFα levels rise early after menopause and remain higher several years after menopause (9, 23), suggesting that TNFα levels are not modified after longer periods of estrogen deficiency.

The source of serum TNFα in estrogen deficient animals is unclear. Although monocytes are considered the primary source of this cytokine; cells of the vascular wall can also produce TNFα. Indeed, in this thesis we are presenting evidence that endothelial cells are able to release TNFα after ANG II stimulation. This is relevant to our aging studies since ANG II tissue levels appeared to be higher in estrogen deficient rats. Although endothelial production of TNFα is lower compared with that from activated monocytes, the large area covered by the endothelium will make of small amounts of

TNFα released from individual cells substantially contribute to total serum levels. Moreover, locally produced TNFα is likely to contribute to altered vascular function.

6.2.2 Effects of estrogen deficiency and TNFα inhibition on vascular function

In our studies estrogen deficient rats had an increase in vasoconstriction, a decrease in agonistic and flow-dependent vasodilation as well as higher levels of shear stress in isolated resistance arteries. These alterations have been associated with the development of vascular disease and strongly indicate that the lack of ovarian hormones, particularly estrogen, is associated with dysfunction of small arteries.

The results of our experiments, explained in the first chapters of this thesis, demonstrate that TNFα inhibition improves vascular function in aged female animals by increasing NO availability; NO modulation of vasoconstriction as well as vasodilation to endothelium-dependent agonist was improved in aged animals treated with Etanercept. Moreover, the vasodilator capacity in these animals was further investigated in experiments evaluating vascular responses to flow, which showed that estrogen deficient animals have a reduction in flow-mediated vasodilation that was also improved by TNFα antagonism. This effect was likely mediated by an increase in NO availability as suggested by a greater modulation of flow vasodilation by L-NAME in Etanercept treated animals. Importantly, the improvement in relaxation was associated with a decrease in shear stress, which at very high levels can contribute to induce endothelial injury.

Previous studies have shown that aging is associated with a decrease in NO availability and a reduction in flow-mediated vasodilation (6, 10, 30, 39). In post-menopausal women these alterations have been associated with a lack of estrogen (6, 16,

40). Results from our studies suggest that TNFα is involved in the alterations of the NO pathway during the state of estrogen deficiency.

The Renin-Angiotensin system has been linked with the pathophysiology of cardiovascular disease (5). Some studies have suggested that estrogen deficiency could be associated with an increase in the actions of ANG II, and thus that ANG II may be a mediator of vascular dysfunction during estrogen deficiency (34-36). In my studies, estrogen deficient animals had a greater expression of ACE, ANG II and AT_1R in mesenteric arteries. It is intriguing however that there were higher levels of ANG II along with higher expression of AT_1R in vessels from aged animals, since ANG II is known to downregulate the expression of this receptor. These results suggest that this effect could be lost with chronic exposure to ANG II. It is tempting to speculate that this effect is NO-mediated and was impaired in aged animals due to a decrease in NO availability. Interestingly, similar results were seen in endothelial cells in which ANG II was unable to downregulate its own receptor (Appendix 1; Gragasin et al)(14).

Interestingly, experiments using a selective AT_1R blocker as well as an ACE inhibitor suggest that the local formation of ANG II is enhanced in estrogen deficient rats and that endogenous ANG II contributes to increase vasoconstriction in these animals. These are novel observations that link the RAS system with vascular dysfunction during estrogen deficiency. Importantly, TNFα antagonism was associated with a reduction in ACE, ANG II and AT_1R expression as well as a decrease in ANG II mediated vasoconstriction, indicating that TNFα may promote the formation and the actions of ANG II during estrogen deficiency.

Some studies have shown that NO and ANG II interact in the modulation of vascular function (14). In fact, NO modulates AT_1R expression (19) and can downmodulate the vasoconstriction to ANG II (20). On the other hand, ANG II is known to increase oxidative stress through stimulating the expression of NAD(P)H oxidase and superoxide anion generation that can inactivate NO (see appendix A; Ferrante et al). Thus, an increase in NO bioavailability by TNFα inhibition could contribute to reduce AT_1R expression resulting in decreases ANG II-evoked vasoconstriction. Likewise, a decrease in the effect of ANG II via AT_1R caused by the treatment with Etanercept may contribute to decreased oxidative stress and NO inactivation, resulting in higher NO availability.

6.3 LIMITATIONS AND FUTURE DIRECTIONS

It is possible that other ovarian hormones or hormones that affect the ovarian function can contribute to the alterations in vascular dysfunction and TNFα levels observed in my studies. In particular, interactions between Follicle Stimulating Hormone (FSH) and TNFα have been described (26, 27). However, since estrogen replacement also reduces FSH, the contribution of this hormone to TNFα levels would be masked by estrogen replacement. Moreover, isolated monocytes from menopausal women spontaneously secrete TNFα, which is reduced solely by estrogen treatment (31). Furthermore, the fact that in our experiments only estrogen replacement was sufficient to revert TNFα levels suggest that the increase in TNFα levels is not dependent on progesterone.

The effect of other inflammatory factors in our studies should also be considered. Indeed, since TNFα is a primary cytokine that modulates immune function and affect the release of other inflammatory factors such as IL-6 (41), it is not possible to rule out that the effects of TNFα inhibition seen in our studies could be mediated by other immune factors. Likewise, the role of other inflammatory mediators on vascular function during estrogen deficiency needs further investigation. Indeed, estrogen can modulate the formation of other cytokines such as IL-1 and IL-6 (32). It is also intriguing that some clinical trials have reported that estrogen replacement may increase serum levels of C-reactive protein (CRP) (28), an inflammatory marker. However, this effect seems to be mediated by a direct action of estrogen in the liver, since dermal preparations of estradiol were not associated with elevated C-reactive protein (42). Moreover, this effect of oral estrogen was not accompanied by an increase in the levels of other inflammatory factors including TNFα (28). Furthermore, whether this effect of estrogen on CRP is associated with an increase in the risk of CVD is unclear.

Although the studies presented in this thesis focused on the NO and ANG II pathways, TNFα can also alter the formation of other vasoactive factors that may play a role in vascular function during estrogen deficiency. For instance, in endothelial cells and VSMC TNFα has been shown to induce the formation of vasodilator (11) as well as vasoconstrictor prostaglandins (17). Interestingly, TNFα potentiates Ang II-induced synthesis of prostacycline in VSMC (4). Thus, a question to address in future studies is whether in aged vessels TNFα makes prevail the formation of vasoconstrictor over vasodilator prostanoids, which our laboratory has shown to occur in the vasculature during aging (3) and estrogen deficiency (8).

The role of interactions between ANG II and TNFα in endothelial function is very intriguing, and our studies provide the foundation for future studies to address the specific cellular mechanisms. A limitation of our conclusions from these experiments in endothelial cells is that they were conducted in specialized human primary cells and there is variability in endothelial cell function across different vascular beds. However, HUVECs express and release TNFα, which make them an appropriate tool to study the regulation of this cytokine, and are also extensively used in the literature as a representative endothelial cell.

Indeed, our experimental observations have been confirmed by recent studies in other cell types. Luchetefeld et al, recently reported that ANG II–induced MMP-2 release is mediated by activation of NAD (P) H oxidase (25), and that ANG II co-localizes with MMP-2 and AT_1R in atherosclerotic arteries (25). Similarly, Wang et al reported an increase in MMP-2 activity in the subendothelial space of aortas from aged animals and colocalized with ANG II (43). These results agree with our findings in endothelial cells as well as with the results of our aging studies and support the broad significance of our mechanistic studies. The role of MMP-2 in vascular function in estrogen deficient animals warrants also further investigation. As demonstrated by our laboratory MMP-2 modulates vascular function by the cleavage of vasoactive peptides such endothelin-1 (12, 13). Thus, it is possible that in aging an increase in ANG-II mediated production of MMP-2 could promote vasoconstriction by cleavage of big endothelin-1.

In addition to the effects of TNFα on endothelial function, TNFα can exert direct effects on VSMC. Indeed, TNFα-induced iNOS expression in VSMC with a subsequent increase in NO production has been implicated in the pathogenesis of hypotension during

septic shock (33). However, iNOS induction requires higher levels of TNFα (~100 ng/mL) (18, 24) than those observed in our studies in aged estrogen deficient animals (~ 30 pcg/mL). Nevertheless, the role of iNOS on vascular dysfunction during aging is unclear. Furthermore, it is also likely that an increase in TNFα during estrogen deficiency may also contribute to enhance vasoconstriction by altering other signaling cascades in VSMC. For instance, TNFα-induced ceramide release from VSMC leads to enhanced-protein phosphatase activity and reduced protein kinase C mediated tonic contraction in aortic rings (21).

6.4 SIGNIFICANCE OF OUR FINDINGS

Our results support the interplay of estrogen with immune factors in the control of cardiovascular function. Although estrogen may affect TNFα transcription directly, future studies should address additional mechanisms that may mediate the increase in TNFα levels during estrogen deficiency (e.g.: Angiotensin II). Moreover, it is also implied from our mechanistic studies that TNFα could be a mediator of other cardiovascular and non-cardiovascular disorders associated with higher levels of ANG II and/or estrogen deficiency. Clinical studies have shown that estrogen deficiency is associated with an increase TNFα levels. The results of the studies presented in this thesis suggest that an increase in TNFα levels in postmenopausal women could result in vascular dysfunction. These are novel mechanistic studies that are the foundation for further research in this field as was recently highlighted by Dantas and Sandberg in an editorial based on our work (7).

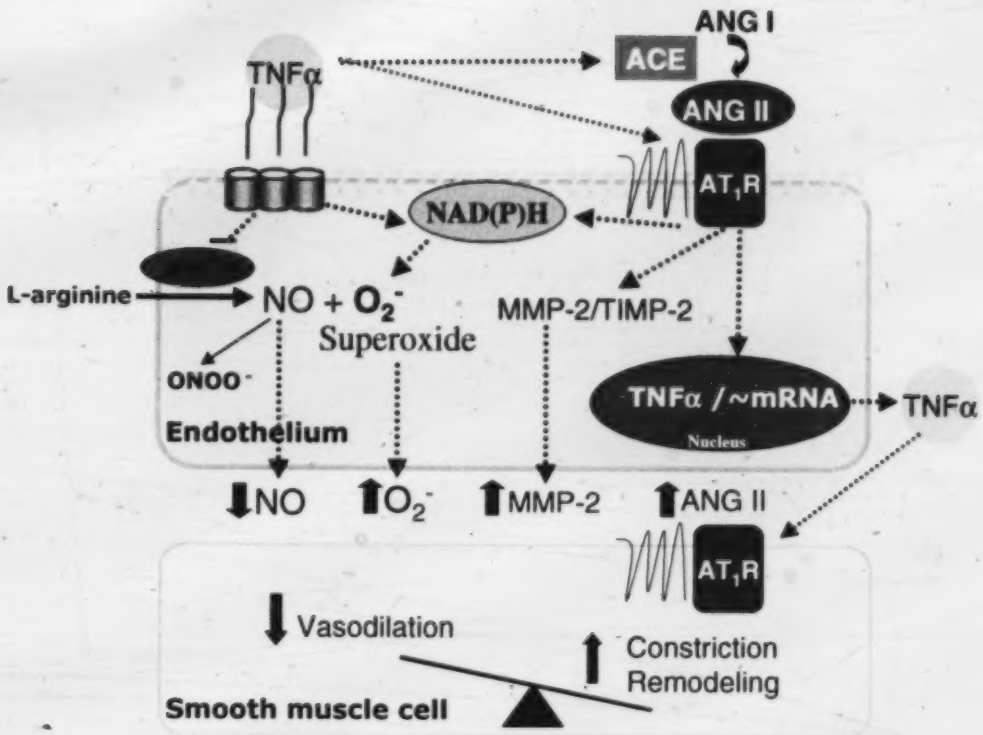

Figure 6.1 Interactions between TNFα and Vasoactive Pathways during aging and estrogen deficiency.

Estrogen deficiency is associated with an increae in TNFα levels. TNFα can reduce NO availability by decreasing eNOS expression or by increasing its inactivatin by superoxide anion. Moreover, TNFα can result in vasoconstriction by promoting the formation of ANG II and its actions through AT_1R, which may also contribute to increase vasoconstriction, vascular remodeling and inflammation through stimulating the local formation of MMP-2 and TNFα indothelial cells.

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APPENDIX I. ABSTRACTS FROM RELATED COAUTHORED MANUSCRIPTS

1. ESTROGEN IMPROVES CARDIAC RECOVERY AFTER ISCHEMIA/REPERFUSION BY DECREASING TUMOR NECROSIS FACTOR-α

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*Equal contribution

1.1 Background

Estrogen has cardioprotective effects from ischemia-reperfusion (I/R). Tumor necrosis factor alpha (TNFα) is a pro-apoptotic cytokine with depressor effects on myocardial function and has been suggested to mediate I/R injury. Whether cardiac TNFα levels are influenced by estrogen status is unknown. We investigated the effect of estrogen on TNFα levels and TNFα receptors in the ischemic heart and its role on estrogen modulation of I/R injury.

1.2 Methods and Results

Ovariectomized Sprague-Dawley female rats treated either with estrogen or placebo during 4 weeks were used in this study. Isolated working hearts were subjected to global ischemia (25 minutes) followed by reperfusion (40 minutes). I/R was associated with a 6- fold increase in TNFα levels in coronary effluent and higher TNFα in LV of estrogen deficient rats, which was decreased by estrogen replacement. Moreover, compared with

estrogen deficient rats, estrogen increased LV expression of TNFR1, whereas TNFR2 was reduced. These changes were accompanied by improved functional recovery, decreased LV apoptosis and reduced myocardial necrosis. To further evaluate the role of TNFα in I/R injury, a selective TNFα inhibitor (etanercept) was used *in vitro* before the ischemic insult. TNFα inhibition improved functional recovery, reduced apoptosis and myocardial necrosis in estrogen deficient animals but did not have a summative protective effect in estrogen-replaced animals.

1.3 Conclusions

These date indicate that estrogen modulates cardiac expression of TNFα and TNFα receptors. Moreover, the cardioprotective effects of estrogen are in part mediated by regulation of TNFα levels in the ischemic heart.

2. Effect of Angiotensin Converting Enzyme I/D Gene Polymorphisms on Serum Levels of inflammatory factors in Hypertensive and non hypertensive Individuals.

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Manuscript under preparation.

2.1 Background

The angiotensin converting enzyme (ACE) gene has been associated to inflammatory conditions and to the risk of cardiovascular disease. We investigated whether the I/D polymorphism of the ACE gene influences plasma levels of C-reactive protein (CRP), IL-6 and TNFα.

2.2 Methods and results

Inflammatory markers and ACE genotype were ascertained in 64 hypertensive and 20 normotensive subjects without history of atherosclerotic disease. High sensitivity ELISA tests were used to measure inflammatory markers and polymerase chain reaction was used for genotyping. Participants were 30-64 years old (mean age 48 ± 9), 37% were male, and 25 % were obese. The prevalence of the D allele was 61% and the polymorphisms were in Hardy-Weinberg equilibrium (p=0.98). Values of CRP, IL-6 and TNFα were log-transformed and subjects with the DD allele (n=31) were compared to those with the II/ID alleles using linear regression. Crude means (95% confidence intervals) for inflammatory markers in individuals with II/ID vs. DD genotype were: CRP (mg/dl): 0.35 (0.26, 0.46) vs. 0.22 (0.16, 0.32); IL-6 (pg/ml): 8.94 (6.56, 12.18) vs. 6.59

(3.75, 11.59); and TNFα (pg/dl): 10.37 (6.20, 17.37) vs. 13.98 (7.36, 26.57), respectively. After adjustment by age, gender, body mass index, hypertension and levels of the other two markers, DD subjects had a 32.7% (1.91, 53.8; p=0.04) lower CRP. This difference in CRP was similar in hypertensive and non-hypertensive subjects (*p*-value for interaction test=0.90). The prevalence of the D allele by quartiles of CRP (lowest to highest) was: 69.0%, 61.9%, 57.1%, and 54.8%. No significant differences were observed for levels of IL-6 and TNFα (p=0.34 and p=0.16, respectively).

2.3 Conclusions

Subjects with the DD variant of the ACE gene have significantly lower levels of CRP than carriers of the I allele. These results indicate that the ACE gene is involved on the modulation of CRP levels. However, the effect of I/D polymorphism seems to be similar in hypertensive and non-hypertensive individuals.

3. ESTROGEN REDUCES ANGIOTENSIN II-INDUCED NITRIC OXIDE SYNTHASE AND NAD(P)H OXIDASE EXPRESSION IN ENDOTHELIAL CELLS.

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3.1 Objective

Angiotensin II (ANG II) has been shown to increase endothelial NAD(P)H oxidase activity, which is a source of superoxide anion that in turn may induce the formation of peroxynitrite. Estrogen (E2) has been reported to have vascular protective effects. In this study, we hypothesized that E2 reduces the ANG II-induced expression of NAD(P)H oxidase and peroxynitrite in endothelial cells.

3.2 Methods And Results

Endothelial cells were cultured and stimulated with ANG II in the absence or presence of E2. Western blots were used to assess nitric oxide synthase (NOS) and NAD(P)H oxidase expression. Immunofluorescence of nitrotyrosine provided evidence of peroxynitrite formation. Our data indicate that ANG II increased the expression of endothelial NOS, inducible NOS, and NAD(P)H oxidase in a dose-dependent manner, which was attenuated by incubation with either E2, superoxide dismutase, or the ANG II type 1 receptor (AT1R) inhibitor candesartan. Estrogen as well as superoxide dismutase also inhibited ANG II-induced AT(1)R expression and nitrotyrosine staining. The effects of

E2 on the ANG II responses were not inhibited by the E2 receptor antagonist ICI-182,780.

3.3 Conclusions

ANG II stimulation of endothelial cells increases expression of NAD(P)H oxidase and NOS, which may contribute to oxidative stress, as evidenced by peroxynitrite formation. E2 inhibits these ANG II effects, possibly through reduced AT1R expression.

4. *ESTROGEN MODULATION OF LEFT VENTRICULAR REMODELING IN THE AGED HEART: INTERACTIONS BETWEEN ESTROGEN AND ANGIOTENSIN II RECEPTORS*

Yi Xu, Ivan A. Arenas, Stephen J. Armstrong, Sandra T. Davidge.

Manuscript published in Cardiovasc Res. 2003 Feb;57(2):388-94.

4.1 Background

To investigate the effects of estrogen on left ventricle (LV) mass and collagen deposition, and on the expression of receptors for estrogen (ER alpha, ER beta) and Angiotensin II (AT_1R, AT_2R) in the heart of aged female rats.

4.2 Methods and Results:

Aged (~ 12 months old) intact (n=7), ovariectomized plus placebo (OVX, n=7), and estrogen-replaced (E2, n=6) as well as young (approximately 3 months old, n=4) female Sprague-Dawley rats were used in this study. After 1 month of treatment, the left ventricular weight/body weight ratio (LVW/BW), changes in myosin heavy chain expression (MHC), matrix metalloproteinase (MMP)-2 activity, the collagen I/III ratio, and the expression of ERs and Angiotensin II receptors in the LV were evaluated. In aged rats, OVX increased LVW/BW associated with a higher expression of beta-MHC isoform, increased collagen I/III ratio, and decreased MMP-2 activity compared to intact rats. Furthermore, the OVX group had a decrease in ERs alpha and beta as well as AT_2R but an increase in AT_1R expression. Estrogen replacement prevented the effects of

ovariectomy on heart remodeling as well as increased further expression of ER beta and decreased AT_1R expression.

4.3 Conclusion

Removal of ovarian hormones increased LV remodeling in the aged rat, which could be attenuated by estrogen replacement. Moreover, regulation of Angiotensin II receptor expression could be a mechanism by which estrogen may modulate heart remodeling.

5. *CARDIOPROTECTION BY CHRONIC ESTROGEN OR SUPEROXIDE DISMUTASE MIMETIC TREATMENT IN THE AGED FEMALE RAT.*

Yi Xu, Stephen J. Armstrong, Ivan A. Arenas, Daniel J. Pehowich, and Sandra T. Davidge.

Manuscript published in Am J Physiol Heart Circ Physiol. 2004 Jul;287(1):H165-71.

5.1 Background

Aging and estrogen deficiency increase the risk for developing cardiovascular disease (CVD). Oxidative stress has also been implicated in the pathophysiology of CVD and in ischemia-reperfusion (I/R) injury. We tested the hypothesis that chronic in vivo estrogen treatment or superoxide inhibition with the SOD mimetic EUK-8 improves cardiac functional recovery after I/R in the aged female rat.

5.2 Methods and results

Sprague-Dawley rats (12-14 mo) were used as follows: intact (n = 6), ovariectomized + placebo (OVX, n = 6), OVX + EUK-8 (EUK-8, 3 mg/kg, n = 6), and OVX + estrogen (1.5 mg/pellet, 60 days release, n = 6). Perfused isolated hearts were subjected to global ischemia (25 min) followed by reperfusion (40 min). Functional recovery after I/R and myocardial protein expression of NADPH oxidase (p22, p67, and gp91(phox)), inducible nitric oxide synthase (NOS), endothelial NOS, and SOD1, as well as nitrotyrosine levels (as a marker for peroxynitrite), were assessed. Compared with OVX, EUK-8 and estrogen markedly improved functional recovery after I/R, which was associated with a

decrease in NADPH oxidase expression and nitrotyrosine staining. However, estrogen increased inducible NOS expression, whereas EUK-8 had little effect. There were no significant changes in endothelial NOS and SOD1 expression among the groups.

5.3 Conclusions

These results indicate that EUK-8 and estrogen improved cardiac recovery after I/R. Given the controversy surrounding hormone replacement therapy, EUK-8 may be an alternative to estrogen in protecting those at risk for myocardial ischemia in the aging population